Physiological and behavioural measures of stress in domestic horses

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By

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ABSTRACT

The welfare of domestic horses has been scrutinised by the scientific community in recent years. Traditional riding and stable management practices have been recognised to be at odds with the physical and behavioural adaptations of the horse. There is, therefore, a growing need to understand how modern horse management can impact on horse welfare.

The first study in this thesis assessed the impact of common management practices on physiological stress in the horse. Faecal cortisol was higher in horses that were stabled and exercised, than turned out to grass with no exercise. The effect of exercise alone was also seen to increase levels of salivary cortisol. No change was seen in cortisol following short-term routine husbandry procedures such as exposure to the sound of electric coat clippers, but it was suggested that this required further investigation. The study confirmed exercise increased stress as reflected by cortisol concentration, and indicated that individual stabling may also contribute to elevated stress. The study recommended that horses may benefit from periods of rest and turn out to grass, to reduce stress levels and improve welfare.

The measurement of stress for the purpose of welfare assessment is, however, best carried out using an integration of both physiological and behavioural measures. Behaviour scores offer non-invasive, quick and easy methods of assessing stress in domestic animals, but have typically been developed using only behavioural assessment of the stress response. In the second study a scale of behavioural indicators of stress was developed using behavioural and physiological measures for the purpose of assessing stress in stabled domestic horses. Principal component analysis of behavioural reactions and changes in salivary cortisol concentration in response to routine husbandry procedures, revealed three meaningful components that were used as the basis to the stress scale. Behavioural reactions to the husbandry procedures were further analysed by a panel of equestrian professionals using free choice profiling, and results were added to the appropriate components. The final scale comprised of four levels of stress (no stress, low,

medium and high stress), and each category was further sub-divided into behaviour scores (BS). The scores represented accumulating levels of behavioural indicators of stress within each stress level, and provided indices of physiological stress. The scale offers an easy to use method of welfare assessment in horses, and reduces the need for additional physiological measures to be taken.

The scale represented a novel approach to measuring stress, and was used in the final study to measure stress in horses stabled individually, group housed, and in horses moved from stabling to group housing. The effectiveness of the scale at measuring stress, was compared to the effectiveness of measures of heart rate variability (HRV) and faecal cortisol at measuring stress in the same horses. Lower levels of stress were recorded in group housed horses as measured by the BS, but measures of HRV and faecal cortisol showed no difference between those stabled or group housed. Stress levels were unaffected by the move to group housing, but BS declined significantly over the three weeks that the horses remained group housed. The physiological measures did not, however, reflect such a decrease in stress. Stress levels were also compared between horses housed in both environments whilst waiting to be fed. Group housed horses had lower stress levels as measured by the BS. Results provided by the BS were supported by relevant literature, and the scale appeared to be more sensitive than the physiological measures which did not yield significant results with the small sample sizes used in the study.

The research confirmed short-term management practices horses are typically exposed to daily, can elevate their stress levels. Further research into which practices put horse welfare at a particular risk, and thus require modification or need to be avoided where possible, is necessary. The findings also suggest horse-owners may need to pay more attention to their horse's stress levels, to avoid repeated or on-going stress that can jeopardise health and welfare. The scale of behavioural indicators of stress would provide a suitable method by which stress could be monitored and thus become a part of horse management.

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Chapter one
General introduction

During recent years there has been an increase in concern over the welfare of horses, and as a consequence the equine industry has come under scrutiny over its management and training practices (McGreevy, 2007). This concern has stemmed from unease over the rigours of certain disciplines such as horse racing (Williams et al., 2001; Pinchbeck et al., 2004), and various high-profile cases that brought dubious horse training methods into question (the use of rapping in show jumping, the practice of hyperflexion or Rollkür in dressage: McGreevy, 2007; von Borstel et al., 2009; inappropriate use of training aids: Goodwin, 2007; McGreevy, 2007), together with an increasing public interest in the care and welfare of animals (Barnett and Hemsworth, 1990).

Members of the equestrian industry who wanted changes to horse management and training, formed groups to discuss and practice alternative techniques. The groups, that were often termed 'horse-whisperers', brought fresh thinking about the relationship between the horse and human. Those first involved tended to suggest that horse trainers ought to 'speak the language of the horse' (McGreevy and McLean, 2010), and so were often emotive and anthropomorphic putting themselves in the position of the horse (Goodwin, 2007). This analysis over current horse management and training practices, did however generate discussion, and acknowledged that horses and humans perceive the world very differently (Keaveney, 2008). This ought to be taken into account when managing and training horses. Change within the equestrian industry had therefore begun, and the need to respect the ethology of the horse began to filter into the equine industry (McGreevy and McLean, 2010).

The emergence of Equitation Science as a discipline (McGreevy, 2007) removed some of the emotive approaches to studying horse welfare and

strengthened the science underpinning the topics considered. Equitation science represented the scientific study of equitation, but did not intend to turn equitation into a science (McGreevy and McLean, 2010). It instead offered a multidisciplinary approach to examining the methods and processes of equitation, horse training and learning (Goodwin, 2007). It also provided a forum to bring together previously disparate groups, such as veterinarians, animal and behavioural scientists, and practitioners, to work towards the common aim of improving the pursuit of horse-riding and raising the standards of horse welfare (Derksen and Clayton, 2007). The Equitation science group recognised that much of the equestrian industry's traditional riding and stable management practices were at odds with the physical and behavioural adaptations of the horse, and that some horses cope with that, whilst others do not (McGreevy and McLean, 2010). As a result, an increasing amount of research into the management and training of horses continues to be conducted, with the ultimate aim being to improve the welfare of the horse.

This study contributes to the increase in concern over the welfare of horses, by providing a valid, non-invasive and economical way of measuring stress levels in horses that will infer their welfare status. An integration of physiological and behavioural measures was used following Ethical Approval (Appendix 1) to develop an incremental scale of behavioural indicators of stress, for use with stabled domestic horses. The scale built on a similar stress-score completed for cats (McCune, 1994; Kessler and Turner, 1997), by combining physiological measures with behavioural analysis. Integrating these measures provided a robust way of assessing stress and welfare (Dawkins, 1998; Moberg, 2000), and represented an approach little practiced due to difficulties associated with correlating physiology and behaviour (Dawkins, 2003). The scale devised therefore represents both a novel approach and a development in the assessment of horse welfare, and thus makes a valuable contribution to this area of research.

1.1 The ethology of the horse

To understand why horses may experience elevated levels of stress in the domestic environment, and thus why their welfare could be compromised, it is

important to consider their ethology. Evolution of the modern horse began some 65 million years ago (Goodwin, 2007). Horses were grazing, browsing animals that roamed for great distances across open plains and lived in herds (McGreevy, 2004). Being part of a herd afforded the horse greater efficiency at carrying out behaviours necessary for survival, such as defending the herd, and finding food (Fraser, 1992). The herd provided the horse with mutual comfort from companionship, as was necessary for this highly gregarious species (McGreevy, 2004). A well defined complex social structure also existed within the herd, maintained by subtle behavioural interactions passed down between generations (Fraser, 1992).

The horse was domesticated during the late Neolithic period about 6,000 years ago (Goodwin, 2007). This advantaged the horse by providing it with food, shelter, veterinary care and protection from predators, therefore improving its chances of survival, but at the same time domestication totally challenged its evolutionary history (Goodwin, 1999). This meant that horses had to try and cope with the constraints of domestication, which included individual or small group tethering and living within enclosures or small buildings (McGreevy, 2004). They were restricted in terms of space and movement, together with social isolation away from the herd, and receipt of food and water at periodic intervals that may not be consistent with how they would have fed in the wild state. Domestication therefore made redundant much innate behaviour that had once been relied upon in the wild (Rivera et al., 2002; Christensen et al., 2002).

Despite this, the modern domestic horse is still a product of its evolutionary past. There is no evidence to suggest that elimination of behaviour essential to survival through successive breeding of domestic horses has taken place (Fraser, 1992). Horses have just had to adapt to modern management through a combination of learning and experience (Fraser, 1992). Many horses show great adaptability to contemporary management practices with few visible problems being evident (Waran, 1997). But for others both behavioural and physiological problems develop, reflecting elevated levels of stress and therefore the welfare of such horses is put at risk.

1.2 The potential causes of stress to domestic riding horses

In Western society horses are frequently an emotional focus for their owners (McGreevy, 2004), and a considerable amount of money is lavished on them (Webster, 2005). This does not however automatically guarantee that domestic horses live in a state of good welfare, and achieve most of their physiological and behavioural needs (Webster, 2005). This is because domestic riding horses are frequently kept in conditions that are incompatible with their evolutionary history (Goodwin, 1999), and therefore their welfare may be jeopardised.

The welfare of domestic animals is influenced by their ability to control events within their environment, and satisfy their needs. If they cannot predict events, they lack a sense of control, and are unable to cope with challenges that may arise, and are unable to fulfil their needs (McBride and Craig, 1985; Tennessen, 1989; Abbot et al., 2003; Webster, 2005). Needs are deficiencies remedied by obtaining resources, or responding to environmental or bodily stimuli (Fraser and Broom, 1997). They are therefore physiological, such as the need to drink; or behavioural, such as the need to gain contact with conspecifics. This does not, however, suggest that wild or feral animals are in a good state of welfare, simply because they have the opportunity to express their behavioural repertoire. This has been illustrated by the constrained behavioural repertoire of feral horses, limited by hunger, disease or injury (Waran, 1997). Being able to perform all behaviour demonstrated by feral animals, does not therefore automatically equate to good welfare for animals in captivity (Veasey et al., 1996). Non-performance of some feral behaviour for example avoiding predators, will not compromise welfare.

The potential causes of stress to domestic riding horses are therefore varied, and stem from the complex relationship between modern intensive husbandry and the horse's ability to adapt successfully to cope with such management (Luescher et al., 1991; Broom and Kennedy, 1993; McGreevy et al., 1995a). A low level of stress, or indeed the absence of any stress in an animal, has been cited as an indicator of good welfare (Mostl and Palme, 2002). This is

because the extreme behavioural and physiological adjustments needed under stressful conditions to enable coping are avoided. This assertion has, however, come under scrutiny, because at times stress can be beneficial such as during exercise where stimulation of the immune system is achieved (Fitzgerald, 1991). An increase in stress cannot, therefore, be automatically correlated with a decrease in welfare (Barnett and Hemsworth, 1990), but repeated episodes of short-term stress or chronic stress may be (Kiley-Worthington, 1997). Within this research stress will suggest a deleterious effect on horse welfare, and its existence at a low level or total absence, will be viewed as a positive factor improving welfare status.

1.2.1 Welfare problems associated with individually stabling horses

The individual confinement of domestic horses in stables is widely practised amongst the equestrian community. Despite concern over the amount of space that should be provided for a stabled horse (see Visser et al., 2008), it is evident that confining horses in stables actually reduces foraging, restricts locomotion, and limits social interaction (Mal et al., 1991). Social isolation denies horses the opportunity to physically touch one another, which has been identified as a particularly serious stressor to them (Luescher et al., 1991; Cooper et al., 2000; McAfee et al., 2002; Visser et al., 2008). It is therefore clear that the confinement and social isolation that stabling imposes, elevates horses' stress levels, and can lead to changes in physiology such as increased defecation, sweating, elevation in heart rate and an increase in cortisol concentration (Bagshaw et al., 1994; Harewood and McGowan, 2005, Visser et al., 2008). Changes in behaviour can also be noted, and these can include increased locomotion through pacing; pawing, aggression, vocalisation, and vigilance, together with feeding disturbances and the establishment of stereotypic behaviour (Bagshaw et al., 1994; Strand et al., 2002; Harewood and McGowan, 2005; Visser et al., 2008). Stereotypic behaviour has been defined as repetitive, relatively invariant and apparently functionless behaviour (Mason, 1991), and will be discussed further, later in this chapter.

Over the long-term, coping with confinement and social isolation can make horses more reactive, by heightening their innate 'flight or fight' response (Harewood and McGowan, 2005; Cooper and Albentosa, 2005; McCall et al., 2006). This suggests that they are experiencing a sustained elevation of stress making them more reactive to other events they are exposed to. Individually stabled horses exposed to novel object tests have demonstrated this reactivity, by exhibiting a higher frequency of defecation, and an increased heart rate with reduced heart rate variability, than pair housed horses exposed to the same tests (Visser et al., 2008). A similar level of reactivity has been seen during training, where stabled horses have shown determination in seeking interaction with other horses during training in close proximity (Rivera et al., 2002, Søndergaard and Ladewig, 2004). The long-term effects of individual stabling have also included poor social skill development in horses (Christensen et al., 2002; Waran et al., 2008; Hartmann et al., 2009), increased levels of aggression (Jørgensen et al., 2009), and greater difficulty in handling (Harewood and McGowan, 2005).

Confinement, and the inability to touch other horses, caused by stabling therefore presents an array of detrimental effects on horse welfare; and so housing horses in pairs or groups maybe preferable (Houpt et al., 1987; Harewood and McGowan, 2005; Visser et al., 2008). This is practiced with other species particularly production animals such as cattle, poultry and pigs, which are often kept in barns and managed as a group or herd. Allowing animals to share space for feeding, drinking and resting enables social interactions to take place, and has afforded them benefits including greater social contact, free locomotion (horses: Lebelt, 1998; McBride and Long, 2001; van Dierendonck et al., 2004), and a reduction in abnormal behaviour (horses: Cooper and McGreevy, 2002; Bachmann et al., 2003; van Dierendonck et al., 2004; Fremstad et al., 2008). In the Nordic countries concern over the effects of stabling on domestic horse welfare has culminated in the funding of a large-scale research project investigating group housing horses, with a view to providing practical solutions to improve horse welfare and associated human safety (Keeling et al., 2007).

1.2.2 Welfare problems associated with feeding domestic stabled horses

Domestic stabled horses are frequently fed highly nutritious diets that are relatively low in fibre (McGreevy et al., 1995a; Rebo et al., 1998, Harris, 1999), and so do not compliment the horse's digestive processes. Horses evolved to consume plant material of relatively low nutritional value, on an ongoing basis to meet their metabolic needs (Fraser, 1992; Zeitler-Feicht, 2004). This trickle-feeding pattern complimented the horse's small stomach, and absence of a gall bladder which causes bile to trickle into the duodenum on a near constant basis (Pilliner and Davies, 1996). Under natural conditions, grazing would be carried out for between 60% and 70% of the day and night (Kiley-Worthington, 1990; Fraser, 1992), enabling constant steady digestion. Domestic diets, therefore, do not complement the needs of the 'trickle-feeder', and the physiological implications of this type of diet over the long-term may include increased gastric acidity, resulting in stomach mucosal damage (Murray, 1992; Murray et al., 1996; Nicol et al., 2002).

The relatively low fibre content of the domestic horse's diet decreases the length of time taken to consume it. The horse lacks stretch receptors within the stomach (Zeitler-Feicht, 2004) so the satisfaction from chewing, or fatigue of the muscles used for chewing, calls a halt to the urge to forage and eat (Zeitler-Feicht, 2004). A reduction in chewing may therefore result in horses not achieving a feeling of fullness, and alternative sources of food such as bedding being sought. This can increase the likelihood of over-eating, weight gain, and in some instances colic (Thorne et al., 2005). Reduced fibre in the diet also reduces saliva production, because saliva is only produced when the stretch receptors of the mouth are activated by chewing or by stimulation of oral tissues (Alexander, 1966; Moeller et al., 2008). A reduction in production of alkaline saliva, reduces its capacity to buffer the stomach's contents, and thus exacerbates acidity contributing to mucosal damage (Hammond et al., 1986; Murray et al., 1996; Orsini et al., 2009).

An additional dietary problem facing domestic stabled horses is that they are often unable to select the forage they eat (Goodwin et al., 2002; Thorne et al., 2005). This is because a single fibre based feed such as hay or haylage is

usually provided as part of the feed ration either in a hay-net or rack. Selection of different forage types, as would occur in the horse at grass, is therefore practically eradicated within the stabled environment, thus reducing this innate behaviour (Goodwin et al., 2002; Thorne et al., 2005).

1.2.3 Other threats to the welfare of domestic stabled riding horses from management practices

Stress caused by inappropriate management of domestic horses, maybe increased by their exercise and training regimes (McGreevy and McLean, 2010). Exercise is frequently provided in a controlled way by riding or lungeing (Cave, 1996), and access to turn out to grass for many horses is infrequent or only for short periods (McGreevy et al., 1995a). Restricted turn-out is especially common amongst competition horses where their value and risk of injury is stated as the main reason for keeping them stabled (Smith, 2006). As a result of long durations spent stabled, horses are likely to exhibit exuberant behaviour when turned out or exercised (Mal et al., 1991). This 'rebound effect' (Christenson et al., 2002) could present potentially dangerous situations for both horse and human.

Moderate exercise levels benefit horses by increasing fitness levels that in turn enhance immune responses (Horohov, 2003). Taking care that horses are fit enough for the work demanded of them, is necessary to avoid physical stress to the body that may potentially suppress immune function (Kurcz et al., 1988; Keadle et al., 1993; Horohov, 2003), and lead to appetite suppression, loss of body weight, poor performance and increased injury rates (Golland et al., 1996; Hamlin et al., 2002). Physical stress arising from training can be combined with the mental rigours of different training methods and competing. The combination of such pressure would increase stress levels especially within younger horses, for example those in training on flat racing yards (McGreevy et al., 1995a; Hutson and Haskell, 1997). If stress levels caused by inappropriate management and training are not controlled, performance is likely to be impaired (Jensen-Waern, 1999). Frequency of illness may also be experienced due to the detrimental effects of stress on immune function

(Bushmann and Baumann, 1991), as noticed on racing yards by the frequency of respiratory infection (Jensen-Waern, 1999).

Finally, domestic horses, especially those used for competitive purposes, are often transported by road, sea or air (Waran and Cuddeford, 1995; Giovagnoli et al., 2002). Transportation can cause additional stress to competition horses (Hodgson and Rose, 1994), arising from confinement when travelling, possible social isolation, noise, vibration, temperature changes, humidity and exposure to exhaust fumes (Waran and Cuddeford, 1995; Giovagnoli et al., 2002). Stress caused by transportation, can contribute to management and training stress, and can lead to both physiological and behavioural changes before, during and after the journey.

1.3 Coping with the domestic environment

The extent, to which animals react to potentially stressful situations within their environment or as a result of their management, is largely determined by their perception of the aversive stimulus (Koolhaas et al., 1999). The animal's perception of the stimulus is influenced by factors that include genotype (Koolhaas et al., 1999), method of weaning (horses: Hoffman et al., 1995; Malinowski et al., 1990; Heleski et al., 2002), age (horses: Waters et al., 2002), gender (horses: Reitmann et al., 2004), experiences (horses: Alexander and Irvine, 1998; Ursin and Eriksen, 2004), social support available (horses: Abbot et al., 2003) and temperament (horses: Visser et al., 2001; Seaman et al., 2002).

Coping with stress involves the normal regulation of body state, together with emergency responses requiring greater energy expenditure (Broom, 1991). Coping actions try to regulate homeostasis, because physiology and behaviour are constantly adjusted to try and maintain normal homeostatic levels (Barnett and Hemsworth, 1990) Such a concept does however need to be approached with some caution, because it suggests that without environmental stressors or challenges, homeostasis and thus good levels of welfare can be guaranteed (Korte, et al., 2007). This is clearly rather simplistic and as a result other theories, such as the concept of allotasis,

stability through change, has been suggested (Korte et al., 2007). This concept may be more accurate in explaining the process of coping, where animals are said to alter physiological variables and behaviour, to meet anticipated demand. Whichever term is more accurate, when coping actions are successful animals show adaptation to their environment, but when coping is only achieved with difficulty or fails, then coping will threaten health, reproduction and survival (Broom, 1991; Webster, 2005). Coping in general, has however been shown to have a reducing effect on stress levels in animals (Wechsler, 1995).

The way in which an animal actually copes with stress has been categorised into two particular coping styles. A coping style can be defined as a coherent set of behavioural and physiological stress responses that are consistent over time, and are characteristic of a certain group of individuals (Koolhaas et al., 1999). To clearly identify a coping style both behaviour and physiology need be measured, and this has been a criticism of some of the previous studies that have identified coping styles in different species, as both parameters have not been measured and thus coping style has not truly been revealed (reviewed by Koolhaas et al., 1999).

Coping styles are broadly based on stress response patterns (Wechsler, 1995; Koolhaas et al., 1999; Koolhaas et al., 2008). The active response is the classic flight-fight escape response, where individuals deal with perceived aversive situations by trying to escape or remove the aversive stimulus. For example, horses that are active copers and are faced with an aversive stimulus may develop stereotypic behaviours to try and adapt to the conditions in which they are kept (Cooper and Albentosa, 2005). These animals are therefore proactive copers as they have developed a behavioural response to the situation. Their physiology will also alter in line with their coping response (Koolhaas et al., 1999; Koolhaas et al., 2008), with high sympathetic reactivity (measured by an increased concentration of adrenaline hormone, an elevation in heart rate response and a decrease in heart rate variability), and low parasympathetic activity. Proactive copers will also show a low HPA-axis reactivity (Koolhaas et al., 1999; Koolhaas et al., 2008).

The other type of stress response is less obvious where animals show few outward signs thus appearing unaffected. These animals are termed passive copers as they do not develop a clear behavioural response to aversive stimuli, and maybe regarded as not actually suffering due to their lack of outward expression. They are clearly less proactive in their coping response, and try to cope by being reactive and flexible to environmental stimuli and thus cope with challenges as they arise (Koolhaas et al., 1999; Koolhaas et al., 2008). If stress persists over time these animals maybe more prone to withdrawing from their environment and retreating into a state of learned helplessness (Murison and Overmier, 1993; Webster, 2005). Their physiology will also alter in line with their coping response (Koolhaas et al., 1999; Koolhaas et al., 2008), with low sympathetic reactivity and high parasympathetic activity. Passive copers will also show a high HPA-axis reactivity.

If methods of coping with the domestic environment fail, or if animals endure ongoing injury, pain, disease, impaired immune function, poor animal husbandry, or barren environments they are likely suffer (Broom, 1991). Suffering is an unpleasant state of mind that disrupts quality of life (Dawkins, 1980; 1990; Gregory, 2004). An animal that is suffering will experience physiological changes and exhibit behavioural indicators (Broom, 1991; Gregory, 2004), both of which are measurable and indicate the likely degree of suffering endured. The emotion being experienced during suffering is more difficult to quantify, and at present is only inferred by physiological and behavioural changes. Behavioural measures have provided the best indication of animal feelings (Dawkins, 2004), but an emerging area of study focusing on the animal's brain may reveal emotional feelings (Panksepp, 2005). There is no doubt that animal emotion may help reveal the extent of suffering endured, as an animal tries to cope with its environment. Animal emotion may therefore have to be included in the assessment of welfare in the future (Mendl and Paul, 2004).

1.4 Assessing stress levels experienced by horses

How to assess animal welfare is a topic still under debate perhaps because there are many definitions of welfare in existence, because the definitions available are so broad, and because there are many different ways to measure it. Broom (1986) defines welfare as (1) a characteristic of the animal, (2) variable over a continuum of poor to good, (3) measurable in a scientific way, (4) failure to cope with the animal's environment, (5) a reflection of preferences, thus providing information on how to improve welfare and, (6) encompassing a variety of coping methods. Based on this definition, any approach to measuring welfare would need to combine a variety of methods to provide a comprehensive picture of how the animal was coping with its environment, and whether its level of welfare could be considered acceptable. In response to public concern over animal welfare in the 1960's the U.K. Farm Animal Welfare Council published "The Five Freedoms" of animal welfare that described housing conditions and care (for a full review see Webster, 2005). These provided a framework by which welfare could be measured, but even this framework was criticised as it was considered subjective in its approach and thus lacking scientific rigour (Korte et al., 2007).

Another approach to measuring welfare has been to use the behaviour of feral animals as a benchmark, by which to infer the welfare of animals kept in captivity. This approach argued that free living animals have the opportunity to express their full repertoire of behaviour, and animals in captivity are denied this so suffer. This approach to measuring welfare may, however, be unwise, as it infers wild or feral animals to be in a good state of welfare because they can express their behavioural repertoire (as mentioned earlier in section 1.2). This, however, does not automatically equate to good welfare for animals in captivity (Veasey et al., 1996), because the expression of some behaviours, such as avoiding predators, will not compromise welfare. The most useful way to use feral studies, therefore, is to identify what is important to the domestic animal in terms of behavioural needs or wants, and to ensure these are available in the domestic environment.

Measuring welfare has traditionally taken on an integrated approach (Moberg, 1987), that has combined physiological and behavioural measures to provide a robust assessment of changes experienced in response to challenges or stressors faced. This approach was adopted in this research, with physiological and behavioural measures taken. The physiological measures used to assess welfare, examined activation of the stress response, which indicates an animal's welfare being at risk (Dantzer and Mormede, 1983; Moberg, 1987; 2000; Mench, 2000). Behavioural measures have provided immediate indicators of the effect of stress (Dawkins, 2004), and have been easy to measure avoiding potentially stressful invasive procedures as necessitated by some physiological assessments. Behavioural measures of stress have focused on short-term, and chronic, behavioural responses, exhibited by the animal when faced with stressful events. Acute behavioural reactions have involved the 'flight or fight' response, and chronic behavioural changes have reflected a failure to adapt to on-going stressors. In between these two extremes abnormal behavioural patterns, unresponsiveness, increased aggression and stereotypic behaviour, have all been measured to indicate diminishing standards of welfare (Barnett and Hemsworth, 1990; Fraser and Broom, 1997).

1.5 The physiological stress reaction

Domestic stabled riding horses are exposed to various potentially stressful situations during their daily management. Stressful situations can be referred to as stressors as they produce a stress response, and can be physical e.g. a trauma, chemical e.g. a reduced oxygen supply, physiological e.g. pain, psychological e.g. fear, or social e.g. isolation from a group (Sherwood et al., 2005). Stressors can be short-lived, producing a short-term stress reaction, or can be chronic leading to a more sustained stress reaction. The impact of either type of stress response can be potentially detrimental to the horse's health and welfare (Broom, 1988), so any measure of stress needs to be effective in assessing stress levels arising from varying situations.

1.5.1 The short-term stress response

During times of short-term stress (based on Sherwood et al., 2005 unless otherwise stated) the sympathetic nervous system (SNS) promotes responses that prepare an animal for strenuous activity. This response is typically known as the 'fight-or-flight' response. In contrast, the parasympathetic (PNS) branch of the autonomic nervous system (ANS) dominates in quiet relaxed situations, when the body is more concerned with 'rest and digest' functions. This dual innervation of the various organs of the body, by the two branches of the ANS, permits more precise control and thus enables an effective response to potentially stimuli.

In mammals, the action of the SNS to stressful situations has an effect on the adrenal glands. The mammalian adrenal glands are positioned above each kidney, and consist of an outer steroid secreting cortex, and an inner catecholamine secreting adrenal medulla. The adrenal medulla is considered as a modified sympathetic ganglion, which secretes hormones directly into the blood on stimulation by preganglionic fibres that originate in the central nervous system (CNS). The mammalian hormones released are the catecholamines, noradrenaline and adrenaline, and their function is to reinforce the activity of the SNS.

The SNS and the catecholamine adrenaline, in particular, are responsible for mobilising the body's resources to support physical exertion in the face of short-term stress. They together exert a wide-spread effect on organ systems that enable the 'fight-or-flight' response. Under their influence the rate and strength of cardiac reaction increases resulting in increased cardiac output, and an increase in arterial blood pressure. This ensures that enough pressure is available in the animal's body to force blood to the organs that are vital in the 'fight-or-flight' response. At the same time, vasodilation in coronary and skeletal muscle enables blood to be received from other vasoconstricted areas of the body. In conjunction with these cardiovascular changes, adrenaline also stimulates the dilation of the respiratory airways during the stress response, to decrease resistance encountered in moving air in and out of the lungs and thus aid the 'fight-or-flight' response.

Adrenaline also exerts important metabolic effects during the stress response. The hormone is particularly responsible for mobilising stored carbohydrate and fat to fuel the stress reaction. Blood glucose levels are increased by gluconeogenesis (the conversion of non-carbohydrate sources such as amino acids to carbohydrate in the liver), and glycogenolysis (the breakdown of stored glycogen to glucose), together with inhibition of insulin secretion thus further raising blood glucose levels. Fatty acid levels in the blood are also increased, through the promotion of lipolysis by elevated adrenaline concentrations. During times of stress the rate of metabolic reactions in the animal's body also alter, with general metabolic rate speeding up.

1.5.2 The long-term stress response

When stressful situations remain for a longer-term than a short-lived phase, various other hormones beyond adrenaline are released in an effort to defend the body against the effects of stress and maintain homeostasis (based on Sherwood et al., 2005 unless otherwise stated). The hypothalamic-pituitary-adrenal (HPA) axis involves the release of glucocorticoids, and measurement of these hormones, namely cortisol and corticosterone, in animals is one of the best indicators of stress. Cortisol in particular is measured in various animals under stressful conditions (dogs: Vincent and Michell, 1992; pigs: Parrot and Mission, 1989; Geverink et al., 1998; de Groot et al., 2000; van der Kooij et al., 2003; sheep: Fell et al., 1985; cattle: Ladewig and Smidt, 1989, and horses: Ralston et al., 1988; Toutain et al., 1995, McBride and Cuddeford, 2001; Covalesky et al., 1992; Stegman and Jones, 1998). The magnitude of the increase in plasma glucocorticoid concentration is generally proportional to the intensity of the stressful situation, so severe stress evokes a greater increase in glucocorticoids than mild stress. The perception of the severity of a stressor depends largely on an animal's individual interpretation of the situation, and this can be influenced by factors including genotype (Koolhaas et al., 1999), method of weaning (Hoffman et al., 1995; Malinowski et al., 1990, Heleski et al., 2002), age (Waters et al., 2002), gender (Rietmann et al., 2004), previous experience (Alexander and Irvine, 1998; Ursin and Eriksen,

2004); social support available (Abbot et al., 2003) and temperament (Visser et al., 2001; Seaman et al., 2002).

When an animal experiences a stressor, the stress response is either directly or indirectly influenced by the hypothalamus (see diagram x). Various areas of the animal's brain such as the amygdala (a structure of the mammalian forebrain believed to store memories of highly emotional events) respond to the stressor and stimulate the hypothalamus. In response, the hypothalamus activates the SNS which causes the adrenal medulla to release catecholamines as previously described. It also stimulates the release of corticotrophic releasing hormone (CRH) which reaches the anterior pituitary by means of the hypothalamic-hypophyseal portal system. This unique vascular link between the hypothalamus and pituitary gland is composed of connecting capillary beds, and provides a critical link between the brain and much of the endocrine system. Increased concentrations of CRH in turn stimulate the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, and finally the glucocorticoid hormone cortisol is released from the adrenal cortex. The posterior pituitary is also stimulated by the hypothalamus, triggering the release of anti-diuretic hormone (ADH) (vasopressin), which increases the permeability of the distal and collecting tubules of the kidneys to water and promotes vasoconstriction.

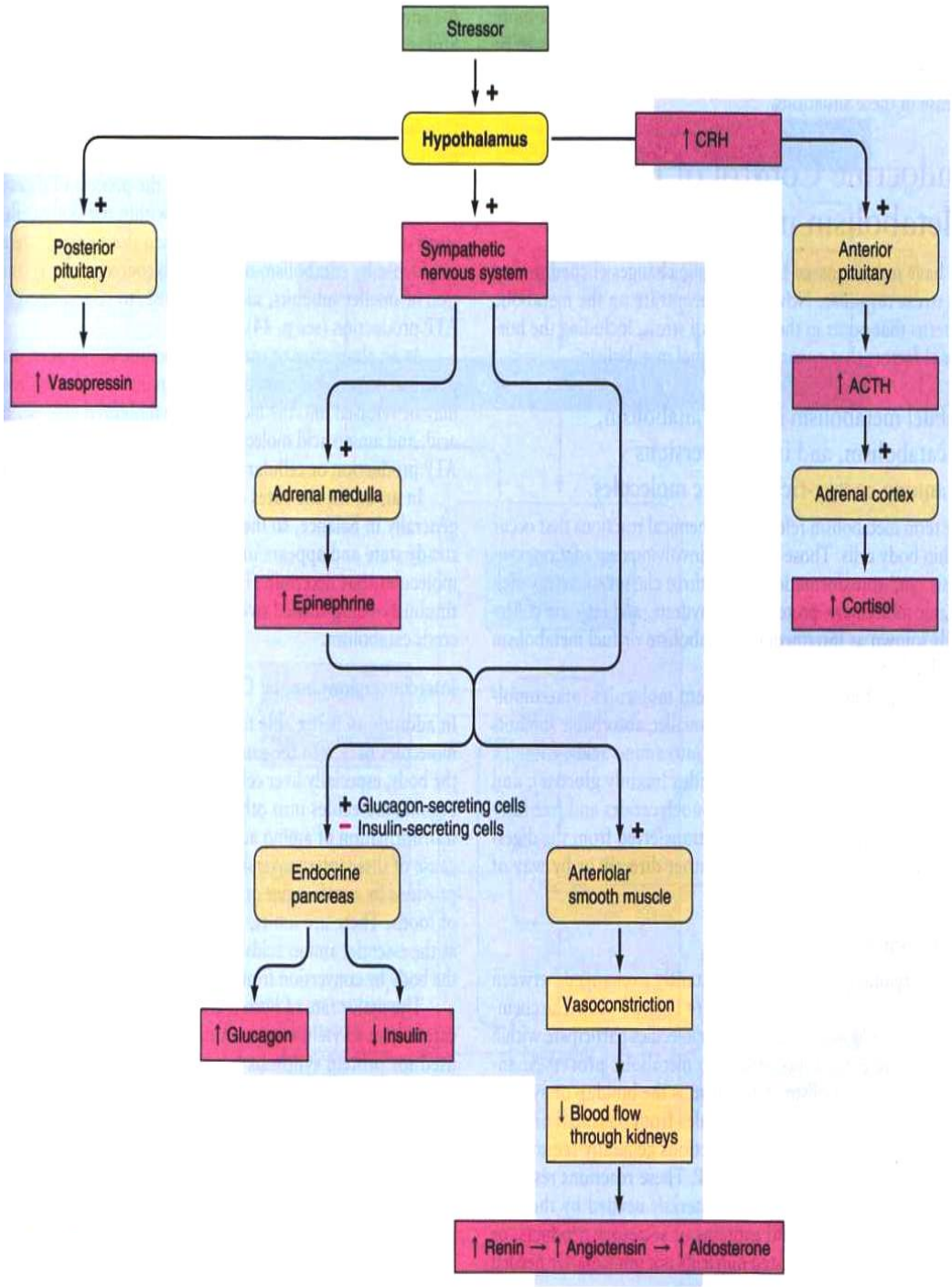

Figure 1.1. Integration of the stress response by the hypothalamus (Sherwood et al., 2005).

Glucocorticoids exert metabolic effects and help in adaptation to stress. Cortisol has a variety of beneficial functions if kept within normal parameters, which for horses is between 0.05 and 0.2μM in blood plasma (Gayrard et al., 1996), or 20ng/ml (Rose and Hodgson, 2000). Cortisol plays an important role in carbohydrate, protein, and fat metabolism; it executes permissive actions for other hormone activities, and helps animals cope with stress. The major difference between adrenaline and cortisol function, is that adrenaline does not promote protein break-down where as cortisol does. During short-term phases of stress it would not be advantageous to break down muscles, but during longer-term phases, the mobilisation of amino acids for gluconeogenesis could be useful.

During chronic stress, however, the beneficial functions of cortisol are outweighed by more detrimental consequences of its on-going secretion. Long-term psychological or physical stress can lead to a suppressive effect on appetite and reduced food intake. This in turn can have a detrimental effect on growth and repair. A reduction in reproductive behaviour has also been noted and, diminished sex hormone concentrations have correlated well with elevated levels of ACTH. Prolonged cortisol secretion has also been seen to suppress the immune system, leaving stressed animals vulnerable to infection.

The detrimental effects of stress on the horse's body brought about by prolonged cortisol secretion; substantiate the need to be able to measure stress levels in horses. By assessing the stress levels of horses, the factors causing any increased levels of stress maybe identified and alleviated, before health and welfare are affected.

1.6 Measurement of cortisol concentration as an assessment of the stress response in various body mediums in the horse

Cortisol is elevated in horses by various stimulants including exercise (Foreman and Ferlazzo, 1996; Marc et al., 2000; Hamlin et al., 2002), semen collection (Lebelt et al., 1996), restraint (Hydbring et al., 1996), transportation (Foreman and Ferlazzo, 1996) and operative procedures (Stegmann and

Jones, 1998). It is also easily measured in various body fluids and excreta (Mostl and Palme, 2002), and can be sampled non-invasively avoiding added stress to the animal. Laboratory assays to establish cortisol concentration are effective, but care are over sampling protocols have to be adhered to, as cortisol is secreted into different body mediums at varying species-specific rates, and via different secretory pathways. Cortisol is also very vulnerable to environmental disturbance, and is affected by physiological and psychological stress. These factors must be accounted for when sampling and interpreting results.

1.6.1 Measurement of cortisol in blood plasma

In blood plasma cortisol is either bound to a specific binding protein, corticosteroid-binding globulin (CBG), or is unbound and free to pass out of blood capillaries (Gayrard et al., 1996; Alexander and Irvine, 1998). The amount of cortisol bound to CBG in the horse is between 70 and 80%, with approximately only 10% being free and able to exert a biological effect (Gayrard et al., 1996). The binding capacity of cortisol to CBG can be altered by factors including the animal's age (Irvine and Alexander, 1987; Alexander and Irvine, 1998), diet (Haourigui et al., 1995) and acute (Fleshner et al., 1995) or chronic stress (Kattesh et al., 1980; Spencer et al., 1996; Alexander and Irvine, 1998). Binding capacity decreases under stressful conditions (Fleshner et al., 1995; Alexander and Irvine, 1998), during exercise (Marc et al., 2000), or when body temperature increases (Toutain et al., 1995). The proportion of free cortisol therefore increases, leading to a greater biological effect on target tissues.

Cortisol secretion in horses is characterised by a well documented diurnal rhythm (Irvine and Alexander, 1994; Alexander et al., 1996; Lebelt et al., 1996). Peak levels are in the early morning prior to wakening (Irvine and Alexander, 1994), and concentrations decline steadily throughout the day to the evening nadir, which can be up to midnight (Stone et al., 2001). The diurnal rhythm is however highly vulnerable to disturbance by various factors such as, environmental changes like alterations in housing (Irvine and Alexander, 1994), social stress (Alexander and Irvine, 1998), exercise

(Foreman and Ferlazzo, 1996; Marc et al., 2000; Hamlin et al., 2002) or anticipation of forthcoming changes that can cause stress or excitement (Alexander and Irvine, 1998). Despite these potential impacts, the diurnal pattern of secretion should be evident in all healthy horses that live in a structured daily routine, where the endogenous rhythm has become entrained (Irvine and Alexander, 1994). The diurnal rhythm of secretion is maintained by a series of ultradian rhythms. These rhythms reflect cortisol secretion from the adrenal cortex which happens in a pulsatile fashion (Fulkerson and Tang, 1979; Irvine and Alexander, 1994; Ingram et al., 1999). The ultradian rhythms prevent any down regulation of the HPA axis, which enables maximum activation of the stress response when necessary (Montford et al., 1993).

When measuring cortisol as an assessment of the stress response, the pattern of cortisol secretion described, must be taken into account. The fragile nature of the diurnal rhythm can potentially affect any assessment of the horse's response to stress (Barnett et al., 1985; Harwood and McGowan, 2005). To overcome this, sampling at the same time daily, and in the morning when cortisol concentrations are higher, and before daily stressors stimulate the HPA axis (Barnett et al., 1985; Harwood and McGowan, 2005) may produce more accurate results. Sampling must also account for a potential lag time between the onset of a stressful event, and the time taken for plasma cortisol to peak. This duration is variable in the horse, and is dependent on the intensity, duration and type of stressor, and the age, experience and temperament of the horse (Hydbring et al., 1996; Marc et al., 2000). Finally, the frequency of sampling can help overcome the effects of the ultradian rhythms. Infrequent sampling may detect cortisol elevation resulting from a peak caused by an ultradian rhythm, rather than the stress response. Calculation of mean concentration over a 100 minute period should reduce such effects (Irvine and Alexander, 1994). Where acute stressors bring about elevation in cortisol, the sampling protocol may have to account for a dip in concentration prior to elevation, due to the 'mopping up' effects of cortisol by CBG (Cook et al., 1996).

1.6.2 Measurement of cortisol in saliva

The unbound portion of cortisol can move out of blood plasma and into saliva by passive diffusion (Eckersall, 1984). Once in the saliva, concentration of cortisol seems to be unaffected by salivary flow rate (Riad-Fahmy et al., 1982). It has not been established however whether cortisol can readily pass back into blood plasma, so it has been suggested that a build up of cortisol in saliva could occur (Pell and McGreevy, 1999). If so, this would make saliva sampling for cortisol concentration problematic, and for animals with oral stereotypies the problem may be exacerbated because the increased oral activity would flush cortisol from the salivary glands (Pell and McGreevy, 1999). These issues have not been further substantiated, and in fact the use of salivary cortisol for studying the stress response has become well established in various species (horses: Lebelt et al., 1996; humans: Vining et al., 1983; dogs: Vincent and Michell, 1992; pigs: Parrot and Mission, 1989; Geverink et al., 1998; de Groot et al., 2000; van der Kooij et al., 2003; sheep: Fell et al., 1985).

One of the reasons for using saliva, instead of blood plasma to measure cortisol, is that concentration of cortisol in both mediums correlate well in animals (Fell et al., 1985; Vincent and Michell, 1992; Cook, 2002). A significant lag time taken for the free fraction of cortisol to move from blood plasma into saliva has not been reported, and so evidence suggests the excretory patterns to mirror each other well in horses (Irvine and Alexander, 1994; Alexander et al., 1996; Lebelt et al., 1996). Salivary cortisol therefore takes on the diurnal pattern of plasma cortisol secretion, together with the ultradian rhythms of secretion. The main difference between plasma and salivary cortisol in animals, has been that plasma concentrations are significantly higher than salivary concentrations (cattle: Negrão et al., 2004), with total amount of salivary cortisol being approximated at 10% of that in plasma (Squires, 2003). This highlights the need for care in sampling to ensure enough saliva is collected for cortisol detection.

Patterns of cortisol response following a stressful event as measured in both blood plasma and saliva have also been very similar. Elevation of plasma

cortisol begins once a stressor impacts an animal, with only a three minute delay recorded in dogs (Vincent and Michell, 1992), and five minutes in horses (Ralston et al., 1988). Time taken for plasma cortisol to peak after the onset of a stressor has been established for the horse at around 30 minutes following exercise stress (Foreman and Ferlazzo, 1996; Marc et al., 2000; Hamlin et al., 2002). Peak salivary cortisol values reported after semen collection from stallions, ranged from 35 to 65 minutes (Lebelt et al., 1996). This suggests the need for saliva sampling to continue for a little longer than sampling from blood plasma, in order that peak cortisol values are sampled. Return to pre-stressor baseline cortisol values in horses have been recorded at just 80 minutes post semen collection (Lebelt et al., 1996), which is faster than following exercise where cortisol can take up to 240 minutes to return to pre-stressor baselines (Marlin and Nankervis, 2002). Sampling protocols therefore need to account for some differences between sampling mediums for cortisol measurement, but demonstrate saliva to be a viable alternative to blood plasma.

Saliva was selected over blood plasma for measurement of cortisol in this study, as blood sampling is a licensed procedure under the Animals (Scientific Procedures) Act 1986. Saliva sampling is less arousing than taking a blood sample (Pell and McGreevy, 1999), and involves minimal restraint of the animals concerned. The non-invasive method of swabbing saliva from the horse's mouth is potentially less stressful to the horse than blood sampling, and so avoids further stimulation of the HPA axis. Any further stimulation of the HPA axis during sampling would lead to increased cortisol levels, and potentially inconclusive results. Saliva sampling was quick and easy, because the introduction of a cotton wool surgical swab into the horse's mouth for saliva collection (Mandel, 1990; Vincent and Michell, 1992; Pell and McGreevy, 1999) caused little concern to horses that were used to having items such as bits introduced into their mouths. Collection of sufficient saliva for effective cortisol detection has been highlighted as a concern though, and particularly when using surgical swabs. Allowing the animal to manipulate the swab for a minimum of 20 seconds however ensured saturation in most cases, and effective cortisol detection. Swabs used in this way seem

preferable to older methods that have included the use of saliva collection bits necessitating longer collection times, and sherbet sweets to stimulate saliva flow (Eckersall et al., 1984) that may potentially affect results. The non-invasive nature of saliva sampling has also enabled saliva to be collected at fixed time intervals before and after an imposed stressor (Negrão et al., 2004). This was useful when assessing the stress response, because the increase in cortisol concentration could be calculated.

1.6.3 Measurement of cortisol in urine and faeces

Urine and faeces both offer the potential for being suitable mediums within which cortisol can be measured, and stress experienced over the longer term gauged. Both enable cortisol to be sampled by non-invasive means, thus minimising the amount of handling, restraint and manipulation of animals necessary. The use of faeces completely removes the need for handling of animals to take place, and so any further stimulation of the HPA axis beyond the effects of the stressor being investigated would be eliminated. Use of these mediums may however be problematic with group housed or wild animals, because the collection of samples on demand may not be achievable or desirable, so both collection and identification of samples may prove difficult. Methods of overcoming these issues must be addressed when devising sampling protocols.

Urine and faeces reflect the species-specific time taken for cortisol to be excreted in the relevant medium following stimulation of the HPA axis. Both media therefore provide an index of circulating cortisol over a time period (Morrow et al., 2000; Mostl and Palme, 2002). Stress experienced over a longer term, rather than just assessment of an acute phase or event can therefore be achieved (Mostl and Palme, 2002). Due to the mediums offering an integrated value of cortisol, concentrations are not as susceptible to the effects of any restraint or handling that may have taken place (Morrow et al. 2000), or the effects of ultradian cortisol secretion.

Urinary cortisol reflects the metabolic fate of plasma cortisol entering the urine via glomerular filtration in the kidneys (Beisel et al, 1964). Urinary cortisol is

secreted as free cortisol, or as conjugates (Toutain et al. 1995). The concentration usually remains below 100ng/ml in resting horses (Ralston et al,, 1988; Toutain et al. 1995; Popot et al., 1997), and is not affected by the sex of the horse, urine concentration, volume or pH (Ralston et al., 1988; Toutain et al., 1995). Variation in urine concentration and volume can be controlled for though, when measuring urinary cortisol for assessment of stress response. This can be achieved by standardising it against creatine, which has a constant elimination rate. Excretion of cortisol in urine does not tend to take on a circadian rhythm in the horse (Toutain et al., 1995), as has been seen in other species such as primates (McCallister et al., 2004).

In horses the predominating excretory route of cortisol is via urine, with less cortisol excreted in faeces (Palme et al., 1996). Cortisol excreted via faeces has been metabolised by the liver, and enters the intestines either free or as conjugates in the bile (Mostl and Palme, 2002). In the digestive system cortisol is either reabsorbed or metabolised by bacteria via deconjugation, oxidation, reduction and formation of ring-A saturated steroids. This means very little authentic cortisol is excreted in faeces, and metabolites of cortisol predominate. In horses 11, 17-dioxoandrostane (11, 17-DOA) is the dominant cortisol metabolite excreted in faeces (Mostl and Palme, 2002).

The time taken for cortisol to pass through the body to be excreted in faeces has been established as 24 hours in horses (Palme et al., 1996). This reflects the intestinal passage time and the absence of any diurnal rhythm. Declines to pre-stressor values have been recorded to take between two and three weeks (Palme et al. 1996). Urinary excretion time for cortisol in horses is less well established and has varied between one and five hours from intravenous administration of cortisol (Palme et al., 1996; Popot et al, 1997). Time taken for urinary cortisol to return to pre-stressor baselines has also varied with reports of between 14 and 17 hours (Ralston et al., 1988), and 10 to 21 hours (Toutain et al., 1995).

Urinary cortisol has been reported as a reliable indicator of HPA axis activity in a variety of species including horses (Ralston et al, 1988; Popot et al, 1997;

Neto et al, 2000), dogs (Beerda et al, 1996), primates (McCallister et al, 2004), pigs (Pol et al, 2002), mice (Touma et al, 2003) and hares (Teskey-Gerstl et al., 2000). Concentrations seem to reflect a similar pattern of elevation and decline that is observed in plasma cortisol as a response to stressful conditions. Despite these merits, use of faeces over urine was chosen within this study as a measure of stress experienced over the longer term. This was because collection of faeces from horses is far easier than urine, and could be achieved in the absence of a researcher thus minimising the impact of human presence on the horse. The option of using an indwelling catheter for urine collection was rejected due to its invasive nature and potential stimulation of the HPA axis. The time course of cortisol excretion in faeces was also more established than for urine, thereby making it easier to assess the effects of the stress response. Faeces therefore offered several advantages over urine as a way of assessing the horse's response to on-going stress. Despite such advantages a number of confounding factors need to be considered in relation to faecal sampling, that include the cortisol assay technique, and biological issues relating to choice of subject animals and their management.

Careful consideration must be given to both the method of faecal sample collection, and the storage of samples. Faecal samples need to be collected as near to their defecation time as possible to minimise microbial metabolism of glucocorticoids that naturally occurs over time (Millspaugh and Washburn, 2004). Such metabolism is accelerated in faecal deposits exposed to moisture from precipitation, and favourable temperatures for bacterial colonization. Cold storage needs to be carried out post collection, and samples must be frozen as soon as possible to preserve glucocorticoid concentrations. The duration of time faecal samples are kept frozen needs to be considered since a decline in faecal glucocorticoid levels have been recorded over a two week period (Lynch et al., 2003). The method of sampling from faecal deposits must be carried out in a way that obtains a representative sample of the total faecal mass (Millspaugh and Washburn, 2004). This is because variability of glucocorticoid concentration exists within the total faeces excreted. Best results will therefore be obtained when

samples have been taken across the faecal mass deposited by the animal, the faecal material has been mixed, and a sample exceeding 0.02g collected. Such a volume has been identified, as very small faecal samples have given rise to disproportionately high faecal glucocorticoid levels.

The choice of animal subjects for faecal sampling requires consideration as variability between individual animals, and their management can lead to differences in faecal cortisol levels potentially affecting results. Gender differences in faecal cortisol concentrations have been noted in a variety of animal species (horses: Palme et al, 1996; mice: Touma et al, 2003; cats: Schatz and Palme, 2001). Findings have shown a higher percentage of cortisol to be recovered from the faeces of males than females. An explanation for this has not been established, but any links between female reproductive status and faecal cortisol concentration, have only been seen prior to parturition when concentrations have been elevated (Huber et al., 2003).

Both season and activity levels have an effect on faecal cortisol in animals. Seasonal effects have resulted in high cortisol concentrations recorded during winter months possibly due to cold stress (Huber et al., 2003). Diet may also contribute to seasonal variation influencing cortisol concentration, especially for herbivorous species like horses. Seasonal differences in grass growth, nutrient value and potential volume of grass consumed will influence dietary fibre consumption. Fibre is a vital component of the equine diet and impacts directly on gut transit time and faecal bulk. Both of these factors are influential on gut metabolism of glucocorticoids (Wasser et al., 1993), so dietary differences between subjects need to be accounted for. Links between activity level and cortisol concentration can be attributed to gut passage times. During times of increased activity gut passage time reduces, thus increasing the rate at which faeces is excreted. The time taken for cortisol levels to peak in faeces following a stressful event therefore decreases (Touma et al., 2003). Management techniques and activity levels need to be monitored in subjects, as these can be influential on the animal's stress response.

1.7 Immunological and biological validation of salivary and faecal cortisol as a measure of stress response

Methods for measuring hormones need to be validated for the chosen medium and species, to establish that they provide reliable estimates of the hormone's concentration within the medium. In this way limitations of the technique can be identified, and the efficiency of extracting the hormone from the chosen medium can be determined (McCallister and Smith, 2001; Buchanan and Goldsmith, 2004).

Enzyme immunoassays (EIA) are analytical techniques used to detect and quantify biomolecules (see Price and Newman, 1997), and are based on a reaction between the molecule of interest e.g. cortisol (the antigen), and the complementary molecule (the antibody). The immunoassay works by using a limited amount of antibody for potential binding to either the antigen under investigation, or to a competitor antigen that has been labelled (cortisol labelled with the enzyme horseradish peroxidase) (CHRP). Both antigens compete for the limited antibody binding sites, and where more antigens are present in their free form rather than labelled (i.e. from stressed horses), they out compete the labelled antigen as they bind more readily. Where a lower proportion of free cortisol (antigen) exists, such as collected from horses that have lower stress levels, labelled cortisol is able to bind more readily to the antibody binding sites. The concentration of the antigen present within the medium under investigation is determined from the proportion of labelled antigen bound to the antibody binding sites.

Validation of an EIA was completed as part of this study, so that levels of excreted cortisol could be measured in horse saliva and horse faeces. Assessment of cortisol concentration in both mediums, served as an index of HPA activity, and provided an indirect measure of stress levels in domestic riding horses.

1.7.1 Assay validation

The immunological validation phase

The validation process comprised of two phases, the immunological validation and the biological validation. Immunological validation is assessed by demonstrating specificity, accuracy, precision and sensitivity of the assay (Diamondus and Christopoulos, 1996; McCallister et al., 2004). The following outline is modified from established descriptions of the criteria for immunological validation given by Reimers and Lamb (1991) and McCallister and Smith (2001).

Specificity is the extent to which the assay measures what it is intended to measure (i.e. cortisol). Other substances within the medium have the potential to interfere during the EIA and affect the specificity. To demonstrate specificity, serial dilutions of the pooled samples (saliva or faeces), and standard solutions of commercially prepared cortisol are tested for parallelism. The parallel inhibition curves demonstrate that the cortisol in the pooled samples, and in the commercially prepared cortisol samples, inhibit binding with the antibody in the same way. This demonstrates that there is no interference by other substances and so samples are comparable. This concludes that the assay is measuring a single component in the pooled samples, and it is immunologically identical with pure cortisol.

The accuracy of the assay is determined by quantitative recovery of known amounts of hormone (cortisol) from the pooled samples (saliva or faeces). If the assay is quantitatively accurate then the quantity of hormone recovered by the assay, should equal the amount added. Precision is expressed as the coefficient of variation (cv), and refers to the agreement between replicate measurements of the hormone in the same assay (intra-assay variation), and between different assays (inter-assay variation). The result indicating a good level of intra-assay precision must be below 10%, and the result indicating inter-assay precision needs to be below 20% (McCallister and Smith, 2001) for the assay to be reliable. Finally, sensitivity of the assay is determined as

the smallest amount of unlabelled hormone (cortisol), which can be distinguished and measured from zero hormone.

The biological validation phase

Biological validation of the assay is determined by investigating whether the assay can detect biologically meaningful changes in hormone levels. Various factors influence the HPA axis in the horse, and therefore influence circulating cortisol concentration. Cortisol levels are highest on waking and decline throughout the day (Irvine and Alexander, 1994; Alexander et al., 1996; Lebelt et al., 1996), thus reflecting a circadian rhythm. This secretory pattern was investigated, to see whether salivary cortisol secretion mirrored plasma cortisol, by identifying a diurnal decrease across the day.

Cortisol also increases in concentration following exposure of the horse to potential stressors that include stabling (Rivera et al, 2002, Christensen et al, 2002), limited turnout to grass (McGreevy et al, 1995b), social isolation (Mal et al, 1991, Harris, 1999, Strand et al, 2002, Harewood and McGowan, 2005), handling of the horse (semen collection: Lebelt et al., 1996; restraint: Hydbring et al., 1996) and exercise (Foreman and Ferlazzo, 1996). Habituation to stress has however been evident in animals; with concentrations of cortisol declining over a five day period where stressors remain present (Redbo, 1993; Morrow et al., 2000, Higashiyama et al., 2007). Detection of these biological patterns of cortisol secretion by the EIA, suggests that the assay is able to detect meaningful changes in HPA axis activity thus demonstrating biological validity. Within this study the effects of exercise, stabling and daily management procedures were all investigated and enabled the biological validation to be completed.

1.8 Measurement of heart rate and heart rate variability as an assessment of the stress response in the horse

The heart is a muscular pump that propels blood around the horse's body (as described by Eckert et al., 2002 unless otherwise stated). It consists of four chambers, two upper atria and two lower ventricles (Figure 1.2). The chambers are connected and guarded by valves, which allow blood to flow in

one direction. Contraction of the heart results in blood being pumped through the chambers and into the circulatory system.

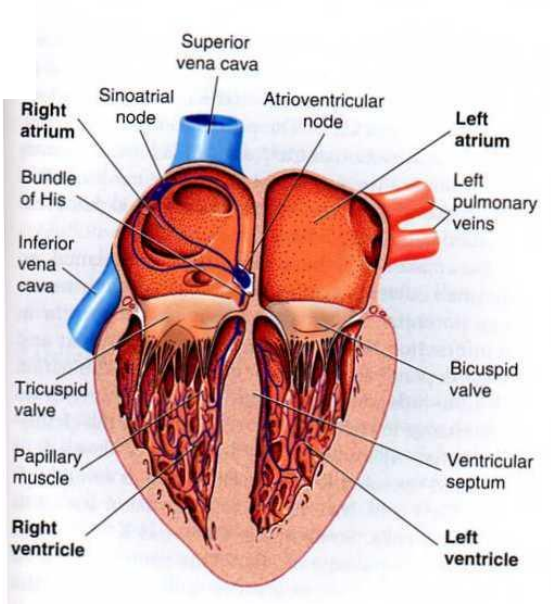

Figure 1.2. Structure of the mammalian heart (Eckert et al., 2002).

1.8.1 Co-ordination of the cardiac cycle

A heart beat consists of a rhythmic contraction (systole) and relaxation (diastole) of the whole muscle mass (Figure 1.3). In the horse approximately three quarters of each heart beat cycle, is spent in diastole (relaxing) enabling the heart to fill with blood (Marlin and Nankervis, 2002). Only one quarter is spent in systole whilst the heart contracts. As the heart works harder, for example during exercise and at times of stress, diastole tends to shorten causing the duration of both diastole and systole to become more even.

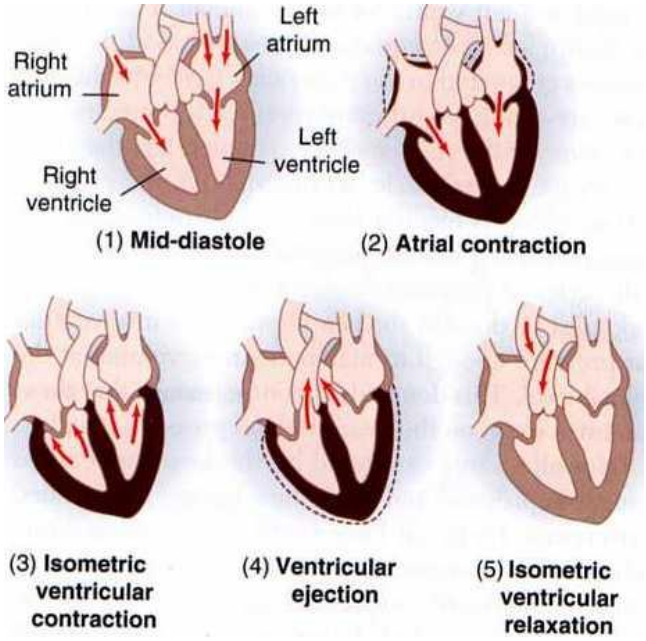

Figure 1.3. The sequence of events during contraction of the mammalian heart (Eckert et al., 2002).

A heart beat is initiated by electrical activity in the pacemaker region of the heart. In most vertebrates this is situated in the sinoatrial node (refer back to Figure 1.3). The pacemaker consists of specialised cells that are capable of spontaneous electrical activity. Electrical activity initiated in the pacemaker is conducted over the entire heart as depolarisation of neighbouring cells. This wave of excitation spreads from the sinoatrial node (SAN) over both atria and through the atroventricular node (AVN) (Figure 1.2) to the ventricles. The wave of excitation slows as it passes through the small junctional fibres of the AVN, allowing the atria to contract before the ventricles. It then passes to nodal, and then transitional fibres, to spread into the bundle of His. This structure subdivides into Purkinje fibres that extend into the myocardium of the two ventricles. The electrical activity causes the two ventricles to contract together.

The contractions of the mammalian heart, cause changes in cardiac pressure and volume, as illustrated in figure 1.4. During the cardiac cycle the following events occur:

1. During diastole the aortic valves are closed maintaining a large pressure difference between the relaxed ventricles, and their outflow channels the aorta and pulmonary artery. The atrioventricular valves are open and blood flows into the ventricles from the venous system.
2. The atria contract raising the pressure within them and blood is ejected into the ventricles. Atrial contraction tops up the nearly full ventricles that have filled with blood directly from the venous system.
3. As the ventricles start to contract ventricular pressure increases and the atrioventricular valves close. This prevents backflow of blood into the atria, and ventricular contraction proceeds.
4. Ventricular pressure rises and exceeds that in the outflow channels. Aortic valves are then pushed open and blood flows out decreasing ventricular volume.
5. As the ventricles begin to relax, their pressure falls, and aortic valves close.

Once ventricular pressure decreases, the atrioventricular valves open, and the ventricles start filling again thus recommencing the cardiac cycle.

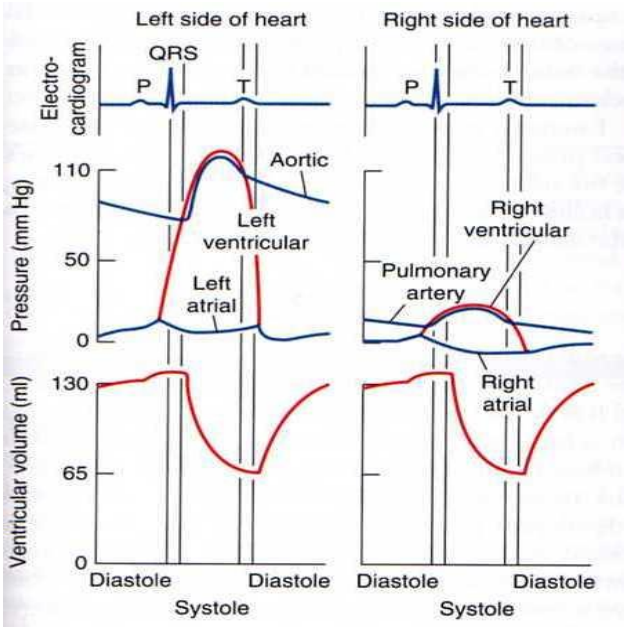

Figure 1.4. Changes in pressure and volume during the cardiac cycle (Eckert et al., 2002).

1.8.2 Recording electrical activity emitted during the cardiac cycle using the electrocardiogram

The electrical currents generated by cardiac muscle during the cardiac cycle, spread into tissues surrounding the heart and are conducted through body fluids (Sherwood et al., 2005). A small portion of this electrical activity reaches the body surface and can be detected using electrodes on the skin. The record produced is an electrocardiogram (ECG), and the wave configuration (Figure 1.5) recorded in vertebrae tends to be comprised of three distinct waveforms:

- The **P wave** representing atrial depolarisation
- The **QRS complex** representing ventricular depolarisation
- The **T wave** representing ventricular repolarisation

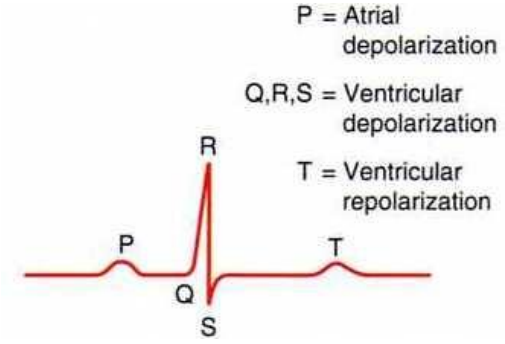

Figure 1.5. The electrocardiogram representing the electrical activity in the heart during a cardiac cycle (Eckert et al., 2002).

In horses, the P wave lasts between 0.12 – 0.14 seconds (Marlin and Nankervis, 2002). An interval (PQ) follows (0.35 – 0.55 seconds: Marlin and Nankervis, 2002), as the electrical activity passes through the AVN, bundle of His and Purkinje fibres, and finally, as the ventricles contract a complex (QRS) is generated (0.10 – 0.15 seconds: Marlin and Nankervis, 2002). The wave (R) of this complex can be large in amplitude, and in comparison to the human ECG trace is a negative wave. This is because the majority of the myocardium of the heart depolarises at the same time due to the deeply embedded Purkinje fibres. This cancels out the wave of excitation travelling

from the base to apex of the ventricles, giving rise to the negative wave (R). A wave (T) completes the ECG trace, as the ventricles re-polarise (or relax).

1.8.3 The effects of the autonomic nervous system (ANS) on HR

The vertebrate heart is innervated by both divisions of the ANS, and together they are able to modify the rate and strength of contraction (as described by Sherwood et al., 2005). The parasympathetic nerve to the heart is the vagal nerve (Porges, 1995; Porges, 2003), which stimulates the SAN and the AVN (von Borell et al., 2007). The vagal nerve reduces cardiac output by decreasing HR, and weakening contractions. These actions are therefore more appropriate in quiet, relaxed situations, where high cardiac output is not necessary.

The effect of the SNS on the heart is to increase cardiac output. The SNS stimulates the heart to improve its effectiveness as a pump, by increasing both HR and the force of contraction. These actions are appropriate for emergency situations such as during the fight-or-flight response, or during exercise. Vagal induced changes in HR tend to be rapid (within five seconds) as opposed to sympathetic nervous system (SNS) regulation. This is partly because SNS changes are mediated by hormonal control (adrenaline and noradrenaline), and that is slower to take effect than nervous responses.

The branches of the ANS therefore work antagonistically to regulate HR. The balance between the inhibitory effects of the vagus nerve, and the stimulatory effects of the SNS, determine HR. In resting healthy animals vagal regulation of HR dominates, but during times of physical or psychological stress, vagal influence decreases and sympathetic influence rises (von Borell et al., 2007).

1.8.4 Regulation of heart rate variability (HRV) in the horse

HRV refers to the time between successive R intervals of the PQRST complex as expressed by the electrocardiogram trace (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). In the horse the t-wave has the potential to be very pronounced, so care must be taken when recording using equipment that only

detects R-peaks of the electrocardiogram that the two waves are not confused (von Borell et al., 2007). HRV data collected in this study was corrected prior to analysis, to try and eliminate any such inaccuracies. HR in resting healthy animals shows a large variation in inter-beat intervals, due to the inhibitory effects of vagal activity on heart rate. Animals subjected to physical or emotional stress however, experience greater SNS activity accelerating HR and reducing HRV (Reitmann et al., 2004).

1.8.5 Use of HR and HRV as a measure of stress response

Measurement of the horse's heart rate provides an assessment of the reactivity of the cardiovascular system to potential stressors. The 'flight-or-fight' response requires the heart to respond rapidly to demand. For this reason horses that are just standing still, can experience sudden increases in heart rate of up to 190 beats per minute resulting from stress (Marlin and Nankervis, 2002). HR is therefore influenced by a variety of factors that include, fear or excitement, the type of exercise being carried out; the environment the horse is in, its health, the breed of horse and the age of the animal (Marlin and Nankervis, 2002). These factors need to be accounted for when using HR as a measure of stress response or work intensity. In this study, use was made of mature riding horses that were managed in a similar way, and received similar work loads to try and reduce the effects of such factors.

HR is therefore a useful measure of the short-term stress response, but may not be suited to measuring longer-term stress. HRV is more preferable for this type of assessment, as it provides a better measure of sympathovagal balance (von Borell et al., 2007). When using HRV for the assessment of stress levels in animals, various factors need to be considered so that results gained are interpreted correctly. Time of day has been shown to influence HRV, with HR being lower at night (Kuwahara et al., 1999a). Despite this, no clear circadian rhythm for HR has been established (Eager et al., 2004). Measurement of HRV in the morning, however, may provide a more accurate assessment of how the animal is coping with on-going stress. This is because data collection needs to be carried out when subjects are resting, calm, and

undisturbed to minimise the effects of short-term stimulants like physical activity, excitement, anticipation of forthcoming events, or other mental arousal on physiological parameters (Langbein et al., 2004; von Borell et al., 2007). Finally, if comparisons of HRV as a way of indicating stress levels are to be made between animals, factors including their age, sex, fitness level, posture, and any physical activity taking place need to be taken into consideration when interpreting results gained (von Borell et al., 2007).

1.8.6 Recording HR and HRV as an assessment of the stress response in the horse

Some of the equipment available for detecting, recording and analysing HR, and thus calculating HRV (reviewed by von Borell et al., 2007), are more suited to recording electrocardiograms (ECG) over long periods, but others can be used over the shorter term. Holter systems (Del Mar Reynolds Medical, Hertford, U.K.; Schiller Switzerland; Rozinn Electronics Inc. U.S.A) are designed for long term ECG recordings. They are very expensive, and are most commonly used in human medicine for assessment of cardiac activity. They have however been used within equine research, to assess the effects of training (Voss et al., 2002), or for clinical purposes such as assessment of pain (Eager et al., 2004).

Other more affordable apparatus suited to field settings that can detect the R-peaks of the electrocardiogram, have been developed by Polar Electro (Öy, Kempele, Finland). Such equipment was devised for sport and sports medicine research, but it is now widely applied to animal behaviour and welfare research. The Polar RS800 was used in this study due to its affordability, suitability for equine use in the field, and because it offered an R-R recording mode that interfaced with the Polar ProTrainer Equine Edition software (Öy, Kempele, Finland). The Polar RS800 used an electrode belt with two electrodes inserted, enabling the electrical activity of the heart to be detected. The transmitter on the belt sent the electrical signals wirelessly to a wrist watch receiver, and the stored data was then downloaded via a Polar Interface (Polar Electro, Öy, Kempele, Finland) to a computer for analysis

using the Polar ProTrainer 5™ Equine Edition (Polar Electro, Öy, Kempele, Finland).

The non-invasive nature of the Polar heart rate monitor makes it a useful piece of equipment for stress research, as it avoids causing any further stress to the horse. Implantable telemetric devices (Data Sciences International, St. Paul, M.N., U.S.A) are available and have been used with various laboratory species (von Borell et al., 2007), but the restraint and need for a surgical procedure to implant such devices, would cause further stimulation of physiological stress related pathways, and the ethics of using such devices are questionable and also require a Home Office Licence. The Polar RS800 offers a number of different features, but simply can record HR at five, fifteen or sixty second intervals, and if HRV is required the wrist watch can be programmed to record at R-R intervals and so records continuously. Despite obvious advantages afforded to the more economic equipment, there are disadvantages that need to be accounted for within methodology.

Positioning of the electrodes incorporated within the electrode belt, and effective contact with the horse's skin to transmit electrical activity can be problematic. One electrode is fitted to the left-hand side of the horse 10cm behind the elbow over the heart, and the other electrode is positioned on the right-side of the horse 10cm behind the withers. Where possible the electrode sites need to be clipped to aid contact, but if this cannot be achieved liberal application of electrode gel is necessary (von Borell et al., 2007). Where only detection of the R-peaks of the power spectrum is being recorded, R-R intervals of only a few milliseconds may need to be discounted in case the t-wave has been recorded. Ectopic heart beats within horses are also common. These small variations in an otherwise normal heart beat, result from a strong parasympathetic influence and tend to be recorded as R-R intervals with large differences (von Borell et al., 2007). Data therefore needs to be corrected for these potential errors using the analysis software to avoid misinterpretation of HRV (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). All data recorded during this study was corrected before analysis.

Various methods of analysis of HRV data can be carried out using the Polar ProTrainer 5™ Equine Edition. Measures of heart beat intervals, together with time and frequency domain measures can be selected from dependent on the type of analysis needed. For this study, the number of heart beats together with the average R-R interval measured in milliseconds, was selected as a suitable measure of stress level.

1.9 Behavioural response as an indicator of stress in the horse

Behaviour provides a direct insight into situations being observed from an animal's perspective (Dawkins, 2006). It is easily observed, an immediate record of welfare that does not require invasive procedures for its assessment (Mench and Mason, 1997; Dawkins, 1980; 2006), and is an essential adjunct to physiology (Dawkins, 2003). Behaviour can indicate the onset of stress, provide symptoms prior to health problems developing, and can tell us what animals really want and how much they want particular things that are missing from their environment (Dawkins, 2003). Behaviour therefore helps us assess levels of welfare, but an animal's developmental history, coping style and current state in terms of health and stress levels, influence how they react thus influencing any welfare assessment being carried out. Such factors led Dawkins (2003) to recommend that welfare assessment is best carried out within the animal's home setting, to truly gain a picture of how they are coping. This advice has been adopted throughout the research reported on in this thesis, as horses were not moved from their home setting, and where possible, or applicable, remained in their own stables.

When animals are constantly or severely challenged, or are unable to mount an effective response they can exhibit a wide range of inappropriate or abnormal behaviours (Webster, 2005). Abnormal behaviour differs in pattern, frequency or context from what is expected from most members of the species, when environmental conditions enable a full repertoire of behaviour to be exhibited (Fraser and Broom, 1997). The term is applied to a variety of behaviours including abnormal postures and movements such as lameness, dog sitting that can be exhibited by pigs, injurious behaviour like tail biting also carried out by pigs, feather pecking by hens, redirected behaviour such as

sham dust bathing by hens, suppression of normal behaviour like in-appetence or learned helplessness, and stereotypic behaviour such as weaving or box walking carried out by horses (Webster, 2005).

The term abnormal behaviour creates controversy in animal husbandry because it is a highly emotive phrase. It may therefore be more of a problem to the people involved in some circumstances, than the animals exhibiting the behaviour. This can lead to some behaviour labelled as abnormal to continue to be perceived as so, even if it actually helps the animals cope with their environment (Cooper and Mason, 1998; Cooper and Albentosa, 2005). Evaluation of abnormal behaviour needs to take into account the cause and effects of the behaviour, by asking why the behaviour started, the costs and benefits of the behaviour to the animal, if the behaviour is really undesirable and to whom, and how best to treat it, if it is perceived necessary to do so (Cooper and Mason, 1998). What ever decision is taken, the presence of abnormal behaviour within the animal's behavioural repertoire still suggests that at some time within its life, welfare standards had diminished leading to the development of behaviour to try and cope with environmental challenges. Any change in an animal's behaviour therefore needs to be taken seriously as it signals a change has taken place within the animal that requires investigation (Waring, 2003).

Stereotypic behaviour is a form of abnormal behaviour that tends to be exhibited when animals are kept in sub-optimal environments, and thus experience stress. It is developed as a way of coping with the challenges or stressors that such environments present (Houpt, 1995; Cooper and McGreevy, 2002; McGreevy, 2004; Webster, 2005). The link between stereotypic behaviour and the domestic environment was made when archaeologists noticed wear, likened to that of crib-biters, on incisors found from Palaeolithic horses (McGreevy, 2004). This suggested that stereotypies developed as a result of the horses' captive environment. This assumption has however been challenged, because sandy soils and certain grasses can produce similar wear on incisors, and because stereotypic behaviour has been recorded in wild Onager, Mountain Zebra and Przewalski horses

(McGreevy, 2004). However, the prevalence of stereotypic behaviour in domestic horses has been reported to be 32.5% in dressage horses, 30.8% in event horses and 19.5% in endurance horses (McGreevy et al., 1995b), with an average level of around 26% across domestic horses in general (Kiley-Worthington, 1983). This suggests that the domestic environment and particular management practices, contribute to the development of stereotypies in domestic horses (McGreevy et al., 1995a and b).

Stereotypies can take on a variety of different forms in the horse (summarised in McGreevy, 2004; Cooper and Albentosa, 2005), and range from crib-biting and windsucking, to box walking, head nodding and wood chewing. These behaviours were previously termed 'vices', and blame for their development was attributed to the horse (Cooper and Albentosa, 2005). They were thought to arise from boredom experienced by horses kept stabled for long durations (Kiley-Worthington, 1987), and the stereotypy therefore served as something to do during this time vacuum. This assumption has since been challenged, particularly because during quiet times of the day horses tend to doze, and bouts of stereotypic behaviour tend to focus around times of activity such as before turning out to grass or around feeding times (Cooper and McGreevy, 2002). This suggests that stereotypic behaviour is caused by frustration, is a way of trying to cope with challenges faced, and is also an attempt to adapt to the conditions in which the animal is kept (Cooper and Albentosa, 2005).

Stereotypic behaviour may therefore actually function as an effective way of coping with stressors experienced within the domestic environment, particularly when stabled. This has been suggested as their prevention has been seen to further elevate stress levels in horses (crib-biting and weaving: McGreevy and Nicol, 1998a & b; McBride and Cuddeford, 2001). Elevation of stress may occur though because the stereotypy has become part of the behavioural repertoire, rather than having to perform the stereotypic behaviour to cope with the environment (Cooper and Albentosa, 2005). The functional significance of stereotypies is therefore still being debated, but some stereotypies do have beneficial functions, and their prevention would be detrimental to welfare. An example of this is the reduction in acidity of the

digestive tract brought about by an increase in saliva flow through crib-biting (Nicol, 1999). This contributes to countering the effects of a predominantly concentrate diet, and the food deprivation experienced between feeds, that can lead to gastric ulceration (reviewed by Cooper and Albentosa, 2005).

1.10 Developing an integrated approach to measuring stress levels in domestic horses

Use of single physiological or behavioural measures to assess stress levels and thus infer welfare status in animals, can be misleading and so an integration of measures is advocated as a more robust approach (Dawkins, 1980; Broom, 1991, Mason and Mendl, 1993; Dawkins, 2003). The difficulty with this approach is, however, how to actually integrate the various measures to provide a meaningful picture of stress levels and welfare status (Dawkins, 2003). There is a lack of information on how different physiological and behavioural measures correlate with each other, and so how increases or decreases over time should be interpreted (Dawkins, 2003). Questions relating to whether some measures should be given more importance within the results remain unanswered.

McCune's (1994) Cat-Assessment-Score was based purely on behavioural measures, but did present a scale of stress levels that physiological parameters could be associated with. It was devised to study the stress reaction of cats, based on five behavioural factors that were suggested to help reduce stress levels of cats housed in single cages during their first 24-hours in an animal shelter (McCune, 1994). The Cat-Stress-Score (Kessler and Turner, 1997) built on McCune's (1994) work by adding postural and behavioural elements to develop a scale from fully relaxed (score one), to extremely stressed (score seven). The scale was applied to boarding cats in a cattery, and successfully assessed the process of adjustment to their new surroundings. The score was a quick, practical, non-invasive method of detecting acute and on-going stress, which offered high inter-observer reliability when used by experienced animal observers (Kessler and Turner, 1997; 1999a and b; Kry and Casey, 2007, Dybdall et al., 2007). It was helpful

in revealing the subjective state of individual animals, and therefore assessing their welfare.

Attempts have been made since the development of the score, to combine physiological parameters with mixed degrees of success. The most widely used physiological measure of stress in cats is urinary cortisol, as urine can be collected easily and non-invasively from the animal (Casey, 2007). Urinary cortisol to creatine ratio, and Cat-Stress-Scores have been found to decrease over time in cats boarding at catteries, but a direct correlation in their decline has not been established (McCobb et al., 2005; Casey, 2007). This is possibly due to differing coping styles adopted by individual cats (Casey, 2007). If an effective method was devised to combine behavioural and physiological measures to form a scale of stress scores, the subjective state of an animal could be revealed more effectively, as these parameters vary differently according to the coping style of individual animals (Casey, 2007).

1.11 Study objectives

Three objectives were carried out to complete this research project:

1. To assess whether managing domestic riding horses in individual stables combined with controlled exercise, would elevate their cortisol concentrations.
2. To develop a scale of behavioural indicators of stress for use with domestic stabled horses for the purpose of welfare assessment.
3. To investigate whether the scale of behavioural indicators of stress was effective at measuring stress levels of horses stabled individually and group housed.

Chapter two

The effects of modern horse management practices on cortisol excretion in domestic riding horses

Abstract

Modern horse management practices, such as individual stabling, have been linked to elevated stress levels in domestic horses. There is, therefore, a growing need to understand how such practices could potentially impact on horse welfare. The aim of the study was to assess the impact of common management practices on physiological stress in horses. We assessed stress levels in two groups of horses that were either individually housed and exercised or turned out to grass with no exercise. In addition we investigated the response of horses to short-term husbandry procedures including the sound of electric coat clippers, short bouts of social isolation, and for the Police horses used in the study the sound of fireworks played from a CD. Physiological stress was quantified non-invasively by measuring levels of salivary or faecal cortisol. Faecal cortisol was higher in horses that were stabled and exercised, as compared to horses that were turned out to grass with no exercise. An effect of exercise alone on cortisol concentration was also observed with higher levels of salivary cortisol, in horses that had been used in group riding lessons compared to horses that were rested and turned out to grass. There was no change in cortisol following the short-term routine husbandry procedures. The study confirmed that exercise does increase cortisol concentration and suggests that individual stabling may also contribute to the raised stress levels. Horses may, therefore, benefit from periods of rest and turn out to grass. Short-term husbandry procedures did not augment stress in this study, but this does require further investigation.

Introduction

A growing number of epidemiological studies have linked modern horse management practices to the development of abnormal equine behaviour (McGreevy et al., 1995; Lụscher et al., 1998; Waters et al., 2002; Bachmann et al., 2003; Parker et al., 2008; Normando et al., 2011). It has therefore been suggested that the psychological well-being of the horse could be improved by understanding modern management practises and how they affect horse behaviour. By providing management practices that acknowledge the relationship between domestic horses and how they evolved, improvements to horse welfare could be made (Henderson, 2007).

As a herd dwelling species that grazed and browsed over great distances, the horse evolved to be a highly social creature, thus requiring social interaction in the domestic environment, together with space to roam and forage (McGreevy, 2004). Individual stabling imposes confinement and social isolation on the horse, and also restricts the time horses can spend foraging. These restrictions, together with the provision of controlled exercise regimes, are clearly at odds with how the horse has evolved (Henderson, 2007, Visser, 2008).

Previous research has identified that the confinement and social isolation imposed by stabling can elevate the stress levels of horses (Luescher et al., 1991; Cooper et al., 2000; McAfee et al., 2002; Visser et al., 2008). This has been reflected by changes in both the horses' behaviour and physiology. Behavioural changes seen in stabled horses have included increased locomotion exhibited particularly by pacing, pawing, aggression, vocalisation, and vigilance behaviour, together with feeding disturbances and the development of stereotypic behaviour (Bagshaw et al., 1994; Strand et al., 2002; Harewood and McGowan, 2005; Visser et al., 2008). Physiological changes have included increased defecation, sweating, elevation in heart rate (HR), and an increase in cortisol concentration (Bagshaw et al., 1994; Harewood and McGowan, 2005; Visser et al., 2008). Stress induced by exercise (for example identified by Malinowski et al., 1993; Zobba et al.,

2011), and from management procedures (for example Normando et al., 2011), have the potential to contribute to the behavioural and physiological changes caused by stabling.

The goal of the current study was to assess whether managing domestic riding horses in individual stables combined with controlled exercise, would elevate their cortisol concentrations i.e. be stressful. It was predicted that cortisol concentrations measured in individually stabled horses receiving controlled exercise, would exceed concentrations measured in horses that were not individually stabled receiving controlled exercise, and instead turned out to pasture and rested.

The study also investigated whether routine management practices, for example exposure to the sound of electric coat clippers, brief periods of social isolation, and for the Police horses used in the study exposure to the sound of fireworks played on CD, undertaken with domestic horses were stressful. It was hypothesised that the management practices would increase cortisol concentrations. This prediction was based on the growing awareness that the way we manage, house and feed domestic horses is suboptimal for the species and can lead to changes in physiology (Harewood and McGowan, 2005; Visser et al., 2008), and behaviour (Bachmann et al., 2003; Parker et al., 2008; Hockenhull and Creighton, 2010).

The physiological stress response is complex, and various measures have been used to assess stress in horses. These have included HR and heart rate variability (HRV) (Reitmann et al., 2004; von Borell et al., 2007; Visser et al., 2008).The glucocorticoid cortisol has been used as an index of hypothalamic-pituitary-adrenal (HPA) axis stimulation (Schmidt et al., 2010; Hughes et al., 2010; Ayala et al., 2011) and thus a direct measure of the stress response.

Cortisol levels can be measured in blood plasma, and noninvasively as the metabolised excreted product in urine, faeces, saliva (Mostl and Palme, 2002), milk (Gygax et al., 2006; Fukasawa et al., 2008), and more recently in

hair (Accorsi et al., 2008; Comin et al., 2011). Non-invasive methods of measurement offer an advantage over the use of blood sampling, as their collection is potentially less disturbing, and so avoids further elevation of stress levels. Measures of stress obtained by non-invasive means may therefore be a truer reflection of the animal's state of welfare. Non-invasive methods do not require a licence for sample collection, and sampling methods do not involve specialised techniques thus rigorous training. There is also less risk of infection through blood borne pathogens. In the current study, we assessed stress levels of horses in response to stabling, exercise and husbandry procedures by quantifying levels of excreted cortisol in saliva and faeces.

Concentrations of salivary cortisol correlate well with cortisol concentrations in blood (Fell et al., 1985; Vincent and Michell, 1992; Cook, 2002). In horses, the excretory patterns of cortisol in both mediums mirror each other and there is a comparatively small lag time for the free fraction of cortisol to enter saliva from blood plasma (Irvine and Alexander, 1994; Alexander et al., 1996; Lebelt et al., 1996). Following exposure to a stressor, cortisol takes between 30 and 65 minutes to reach peak concentration the horse (exercise stress: Marc et al., 2000; Hamlin et al., 2002; semen collection: Lebelt et al., 1996), and so cortisol measured in saliva is well suited to the assessment of the effects of short-term stressors.

Faecal cortisol, however, is suited to reflecting stress experienced over the longer-term in horses (Harper and Austad, 2000) since cortisol takes 24 hours to be metabolised and excreted (Palme et al., 1996), and thus provides an average level of circulating cortisol rather than a point in time sample as provided by cortisol in saliva. Faecal cortisol is, therefore, less vulnerable to short-lived environmental stimulants. In the current study we used levels of salivary cortisol to asses exercise stress and the response to short-term husbandry procedures. Titres of faecal cortisol were quantified to investigate stress levels in stabled exercised horses and horses turned out to pasture and rested.

Methods

Subjects and husbandry

Subjects were 37 mature crossbred domestic riding horses kept at four separate locations (n=11 mares and n=26 geldings see Table 1). Data was collected from 24 of the horses for the purpose of validating the salivary cortisol and faecal cortisol assay (Table 1).

On weekdays, horses were housed in individual stables within an American Barn on either straw or shavings bedding. All had access to hay or haylage and water at all times, and received two concentrate feeds daily at 0700h and 1600h. The stables enabled restricted tactile contact with neighbouring horses through vertical bars and visual contact was enabled over half doors. At weekends the horses were turned out into grass paddocks enclosed by electric fencing, in small groups of up to five horses that remained the same throughout the three week study. All horses received a maximum of two hours of exercise in group riding lessons daily and remained in their usual daily management routine throughout the study.

Experimental design for the assessment of the effects of stabling and exercise on domestic horses

The study was conducted over a three week period. A sample of 18 horses were individually stabled and exercised for a maximum of two hours daily during the weekdays of three consecutive weeks. On the Friday evening of each of the three weeks at 1700h, all of the horses were turned out to grass and rested from exercise for the weekend. They were brought back into their stables in preparation for the working week each Sunday evening by 1600h.

Faeces sample collection and extraction

Faeces were collected from each horse every time they defecated (including during exercise) between 0800h and 1700h on Tuesday and Wednesday over the three week period, and over the three weekends. This enabled the effects of stabling and exercising horses during the week, and turning them out to grass and resting them at the weekends to be compared by measuring faecal

cortisol concentrations. Weekdays were selected at the start of the week to avoid potential habituation to stabling and exercise. Faecal samples were therefore collected on Tuesdays and Wednesdays to accommodate the 24-hour lag time for cortisol excretion in faeces in the horse (Palme et al., 1996). Faecal samples were collected on Sundays and Mondays to represent weekend days again because of the 24-hour lag period.

Faecal samples were collected as soon as the horses had defecated and were taken by removing a pinch of the faeces from the centre and each sides of the pile in an attempt to gain a representative sample of any hormone present. Gloves were worn during sample collection. This method was adopted as it is known that hormones can be unevenly distributed in the faecal bolus for some species (see Millspaugh and Washburn, 2004). Samples were then placed into sterile 20ml plastic screw top containers, labelled, and stored on ice until frozen at -20°C the same day to await cortisol extraction.

Following defrosting 10g of each faecal sample was weighed, placed in a foil container, and dried at 40˚C in a drying oven for two days. Each dried sample was then ground in a pestle and mortar to increase the surface area of the faecal matter and sifted through a fine wire mesh to remove fibrous material. A 0.2g sample of the resulting powder was mixed vigorously with 3ml of 90% methanol (M/4053/17, Fisher, Loughbourgh, UK) through agitation using a vortex for one minute, and then by being shaken for three hours in a shaking incubator at 25˚C (Stuart Scientific, UK). The samples were centrifuged using a Sorvall T.C. centrifuge (Thermo Scientific, Basingstoke, Hampshire, UK) at 800g for 15 minutes. The resulting supernatant was removed and poured into another glass test tube, and the methanol was evaporated off using compressed oxygen free nitrogen gas (N_2) administered using a Pierce Reacti-Therm Heating Module (Pierce, Rockford, Illinois, USA) for approximately 40 minutes at 40^{o}C.

The samples were then reconstituted in 1ml of EIA phosphate buffer saline solution (PBS) (PBS – 5.42g $NaH_2PO_4H_2O$, 8.66g Na_2HPO_4 [anhydrous], 8.7g

NaCl, 1.0g BSA [RIA Grade Albumin Bovine] and 1L dH_2O, pH 7.0, stored at 4°C). To ensure mixing; test tubes were agitated using a vortex for 3 minutes, and then the solutions were frozen at –20˚C to await assay.

Experimental design for the assessment of the effects of exercise on salivary cortisol concentration in domestic horses

A sample of eight horses was kept individually stabled during the weekdays of one week with no access to pasture turn out, and was used to assess the effects of exercise on salivary cortisol concentration. Light to medium level exercise was undertaken by the horses daily for one hour periods in group riding lessons, carried out in a 40m by 60m sand and rubber surface outdoor arena.

Saliva sample collection to assess the effects of exercise on cortisol concentration

Saliva was collected from all horses (n=8) at 30 minute intervals (except during exercise) starting at 0900h and finishing at 1600h over three consecutive weekdays (Tuesday, Wednesday, Thursday) during the week. Salivary cortisol concentration was recorded in the 30 minutes pre and 30 minutes post exercise for all horses during all exercise periods. Mean salivary cortisol concentrations before and after exercise were compared to assess the effects of exercise on cortisol concentration.

Experimental design for the assessment of the effects of routine management practices on cortisol concentration

A sample of 10 horses underwent 10 minute routine husbandry procedures whilst loose, individually housed in their stables with access to hay and water. The husbandry procedures included exposure to the sound of electric coat clippers from an adjacent stable (n=5 horses); being caught from the field and socially isolated in a stable for 10 minutes before neighbouring horses were returned to their stables (n=5 horses); and for the Police horses that participated in the study, exposure to a CD playing the sound of fireworks (n=3). This procedure was used by the Mounted Police as part of riot training

with their horses, and represented a more extreme stressor that some horses may experience.

Saliva sample collection to assess the effects of management procedures on cortisol concentration

Saliva samples were collected immediately before the 10 minute husbandry procedure commenced, half way through the procedure (at five minutes), and then at the end of the ten minute procedure. Saliva samples were then collected every ten minutes after the procedure finished up to 40 minutes in expectation of a peak cortisol response at 20 to 30 minutes post-stressor (Hydbring et al., 1996; Marc et al., 2000).

Saliva collection and extraction

Saliva was collected whilst horses were stabled individually, and use was made of only a head collar and rope for restraint when necessary. Saliva was collected using sterilised flexi-swabs (Medical Wire & Equipment Co (Bath) Ltd) that were introduced into the corner of the horses' mouths, first on the horse's left and then on the horse's right. The horses were not trained for the swabbing procedure and instead were allowed to manipulate the swabs using their tongues for 20 seconds per introduction of the swab. This method was adopted to try to saturate the swabs with saliva, in order that sufficient medium was available for cortisol measurement. The swabs were then placed into sterile 20ml plastic screw top containers, labeled and stored on ice until frozen at -20°C the same day to await cortisol extraction.

To extract the saliva from the cotton wool swabs, they were centrifuged using a Sorvall T.C. centrifuge (Thermo Scientific, Basingstoke, Hampshire, UK) for two minutes at 800g. The supernatant was then centrifuged using a Hettick Mikro 20 centrifuge (Tuttilgen, Germany) at 15,000g for two minutes. The supernatant was taken off using a pipette and frozen to await analysis.

Cortisol Enzyme-Immunoassay (EIA)

Faecal and salivary cortisol concentrations were quantified using a modified version of an EIA described by Smith and French (1997).

Immunological Validation

The assay was immunologically validated for quantification of faecal and salivary cortisol in domestic horses using a representative sample pool of faeces and saliva respectively from mature mares and geldings see table 1 (Diamandus and Christopoulos, 1996).

Cross reactivity of the cortisol monoclonal antibody (R4866 raised against a steroid bovine albumin (BSA) in rabbit (Munro and Stabenfeldt, 1985) was 100% with cortisol. Cross reactivity with similar steroids was 9.9% with prenisolone, 6.3% with prednisone, 5.0% with cortisone, 0.7% with corticosterone and <0.3% with various other steroids (Munro & Stabenfeldt 1985). Assays were carried out in triplicate for the faecal pool, and duplicate for the saliva pool. Linear regressions of the displacement curves of serial dilutions of cortisol standard and the mixed faecal and saliva pool did not differ significantly inferring parallelism and assay specificity (faecal pool: ANCOVA; $F_{2,1} = 3.26$, ns; salivary pool: ANCOVA; $F_{1,14} = 0.13$, ns). Recovery of the standards (halving dilutions in the range 10 – 0.33ng/ml) added to a 1:5 dilution of a mixed faecal pool was 110.94 ± 11.81% (r_2=0.92, P<0.01, y=19.80x-82.07, n=3). Recovery of the standards added to a 1:2 dilution of a mixed saliva pool was 86.55 ± 7.98% (r_5=1.00, P<0.001, y=0.67x-0.19, n=5). Intra-assay coefficients of variation of replicates for the faecal pool was 8.86% (n=11), and for the saliva pool was 1.80% (n=35). Inter-assay coefficients of variation for the faecal pool was 32.36% (n=5), and for the saliva pool 6.55% (n=6). Sensitivity of the assay was determined as 0.16ng/ml.

Biological Validation

For practical and ethical reasons blood plasma measurements and / or an adrenocorticotrophic hormone (ACTH) challenge were not used to establish whether the faecal and saliva assays reflect adrenal activation (e.g. Setchell et al., 2010). We were able to validate the faecal assay biologically by demonstrating that cortisol levels as measured by our assay increased following exposure of horses to stabling and exercise as predicted.

Biological validation of the salivary assay was investigated by exploring circadian variation in cortisol excretion in horses (n=15) between 0900h and 1600h. For the purpose of our analyses we divided the day into seven hour-long periods: 0900 - 0959h, 1000 - 1059h, 1100 - 1159h, 1200 – 1259h, 1300 – 1359h, 1400 – 1459h and 1500 – 1600h. In each time slot two saliva samples were collected each individual, and the hourly mean cortisol concentration entered into the analyses. For four time slots only one sample was available from eight of the horses.

Statistical analysis

All cortisol concentrations were explored for normality and homogeneity of variance by means of Shapiro-Wilks' and Levene's tests respectively, and where necessary log-transformed. All statistical tests were two-tailed unless stated otherwise, and alpha was set at 0.05.

The effects of stabling and exercise, on faecal cortisol concentrations, were investigated by comparing mean weekend concentrations (when horses were turned out to grass and rested), to mean weekday concentrations (when horses were stabled individually and exercised) using an independent samples t-test. An independent samples test was chosen, because availability of the horses meant that the same animals could not be collected from during the week and at weekends. This study also provided biological validation for the faecal cortisol assay.

The effects of exercise on salivary cortisol concentration was investigated by comparing mean concentrations of salivary cortisol in the 30 minutes before, and the 30 minutes after exercise (N=8) using a paired samples t-test. Changes in salivary cortisol concentration over the 10 minutes of the husbandry procedures, and the 30 minutes post exercise period were investigated using a repeated measures ANOVA.

Changes in levels of salivary cortisol across the day were explored using repeated-measure ANOVA.

Results

The effects of stabling and exercise on faecal cortisol concentration

Faecal cortisol concentration increased significantly in the presence of predicted environmental stressors stabling and exercise, as compared to when the stressors were absent (turn out to grass and rest) (Figure 1. $t_{25} = 2.256$, $P=0.03$). Increases in levels of faecal cortisol as measured in our assay in response to presumed stressors provided biological validation of the EIA.

The effects of exercise and routine husbandry on salivary cortisol concentration

Salivary cortisol concentration was significantly higher in the half hour post-exercise, than in the half hour pre-exercise in horses that had undertaken one hour of exercise in a group riding lesson (Figure 2. $t_{7}=3.452$, $P=0.01$).

It was found, however, that there was no overall change in salivary cortisol concentration over the 10 minutes of the routine husbandry procedures, and during the following 30 minutes ($F_{5, 45}=1.074$, ns).

Biological validation of the EIA for measurement of salivary cortisol

Biological validation of our EIA to quantify salivary cortisol was achieved by demonstrating a diurnal change in cortisol concentration across the day from 0900h to 1600h (Figure 3. $F_{7, 98}$=2.58, P=0.046).

Discussion

The effects of modern horse management practices on cortisol levels

It was hypothesised that modern horse management practices would increase cortisol concentrations in domestic riding horses. It was predicted that cortisol levels would increase in horses that were stabled and exercised, as compared to horses that were turned out to grass and rested. Faecal cortisol concentrations supported this prediction by showing a significant increase in cortisol during the week when horses were stabled and were being ridden, as compared to the weekend when they were turned out to grass and rested. In order to separate the effect of being stabled versus being exercised on cortisol levels we investigated the effect of exercise only on HPA activity by comparing levels of salivary cortisol pre and post exercise. As expected, cortisol titres were higher post exercise confirming the results of other studies that found exercise to increase cortisol concentrations (Covalesky et al., 1992; Malinowski et al., 1993; Toutain et al., 1995; Foreman and Ferlazzo, 1996; Marc et al., 2000; Hamlin et al., 2002; Zobba et al., 2011).

It can, however, only be speculated that stabling contributes to the elevated cortisol levels measured in the individually stabled horses that were being exercised during the weekdays. It is well known that the confinement and social isolation (such as that enforced by individual stabling), elevates the stress levels of stabled horses (Mal et al., 1991; Luescher et al., 1991; Cooper et al., 2000; McAfee et al., 2002; Visser et al., 2008). It therefore seems reasonable to assume that stabling contributed to weekday stress experienced by horses in this study.

Housing horses in social groups combined with rest appeared to reduce cortisol levels in our subjects. A decrease in stress levels has been recorded in horses when housed in groups and thus able to socialise (Christensen et al., 2002; Bachmann et al., 2003; van Dierendonck et al., 2004), and in grouped horses provided with space to move around and exercise freely (Rose-Meierhöfer et al., 2010; Werhahn et al., 2011). This further substantiates the assumption that stabling contributed to the increased cortisol concentrations measured in study horses during the week, and that turn out to pasture in the absence of controlled exercise lowered cortisol levels.

It was also hypothesised that routine husbandry procedures horses may experience as part of their daily routine, such as brief periods of social isolation and being exposed to noise from machinery such as clippers, could be stressful to them. Such a claim could not be supported in this study, as salivary cortisol concentrations remained unaffected by the husbandry procedures undertaken. This may have been because the husbandry procedures were not stressful to the horses concerned, possibly because the majority of the study horses were exposed to various husbandry practices on a regular basis and were therefore used to them. Further research would be necessary, however, to generalise this finding to the wider horse population, as the husbandry procedures may have been stressful to other groups of horses. Further work focusing on the frequent short-term stressors that horses have to contend with on a daily basis would be useful as the majority of behavioural problems in horses have been linked to management issues such as stabling and feeding practices.

The increased levels of faecal and salivary cortisol measured following exercise stress, and the assumption that individual stabling also contributes to elevating cortisol concentrations, suggests that that modern management practices do elevate stress levels experienced by domestic riding horses. The stressful effects of modern management techniques can be short-lived, for

example stress caused by an hour's riding lesson, or they can last over the longer term for example from the absence of social interaction. Short-term stress will elevate cortisol concentrations but this may not be detrimental to the horse's health and welfare, because over the short-term glucocorticoids improve levels of fitness by energy mobilisation (Mostl and Palme, 2002). If, however, stress is prolonged over an extended period elevation of cortisol can result in reduced fitness of an animal characterised by factors including, immunosuppression, reduced reproductive potential, diminished growth, appetite suppression, and changes in behaviour (Moberg and Mench, 2000). It is therefore important to know what constitutes prolonged stress for horses and modify or remove such factors where possible. Results of the current study suggest that stabling may constitute an on-going stressor for horses and exposure to this housing practice should be minimised.

Accurate measurement of stress experienced over the short and long-term is vital in ensuring the horse's well-being. The study findings confirmed that measurement of cortisol in faeces offers an effective, non-invasive means of assessing stress experienced over the longer term. Despite offering several advantages as a non-invasive means of measuring the effects of stress, there are difficulties related to faecal sampling and cortisol measurement. Appropriate precautions were taken in this study to minimise the effects of these. Steroid hormones can accumulate in the outer layers of faecal balls (Palme et al., 1996; Wasser et al., 1996), so a representative sample of total faecal mass exceeding 0.02g (Millspaugh and Washburn, 2004) was obtained to try and overcome inaccuracies in measurements. Faeces was also collected as near to excretion time as possible to avoid microbial metabolism of glucocorticoids (Millspaugh and Washburn, 2004), and samples were frozen and assayed as soon as possible (Lynch et al., 2003). Any study assessing cortisol concentrations will be presented with a variety of confounding factors that may not be totally controlled for and may potentially affect the results. These may include sex of the animals used (Palme et al., 1996; Schatz and Palme, 2001; Touma et al., 2003); age, hormonal status (Millspaugh and Washburn, 2004) and the animals' diet (Wasser et al., 1993).

The use of mature stabled riding horses which were managed in a similar way reduced the impact of some of these variables on this study.

The study findings also suggest that salivary cortisol provides an effective measurement of pituitary-adrenal function and short-term stress. This was evident by the increased levels of cortisol seen after sixty minute group riding lessons. Individual differences in excreted cortisol were, however, greater in saliva than faecal measures. Salivary cortisol concentrations ranged between 0.37ng/ml to 2.65ng/ml. These results were, however, concurrent with results in other studies (1.5ng/ml and 3.5ng/ml: Pell and McGreevy, 1999; 0.5ng/ml and 1ng/ml: van der Kolk et al., 2001; 1.5ng/ml and 3.5ng/ml: Harewood & McGowan, 2005). Salivary cortisol may, therefore, be best used in a repeated measures design as was completed in this study, to overcome the effects of individual variation.

Conclusion

The study showed that exercising horses increases their cortisol levels. It also suggested that stabling horses individually may contribute to increased cortisol concentrations. Domestic riding horses may therefore benefit from periods of free exercise at pasture, as cortisol levels were seen to decline in study horses kept in this way. The routine husbandry practices carried out in this study did not affect cortisol concentrations, suggesting that the procedures were either not stressful to the horses concerned, or that the horses had habituated to them so they no longer caused an HPA axis response. Further research into the effects of routine management procedures on cortisol concentrations is therefore necessary.

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Table 1.

	Location	Age	Gender	Breed/type	Horses used for immunological validation	Horses used for biological validation	Horses used for husbandry procedures
1.	1	6	Mare	Cob	s	s & f	
2.	1	11	Gelding	Welsh x Cob	s	s & f	
3.	1	11	Mare	TB X			✓
4.	1	10	Mare	TB X	s	s	
5.	1	14	Gelding	Fell Pony	s	s & f	
6.	1	10	Mare	Clydesdale	s	s	
7.	1	4	Mare	Welsh X	s	s & f	
8.	1	6	Mare	Welsh X	s	s & f	
9.	1	10	Gelding	Welsh X	s	s	
10.	1	9	Gelding	Welsh X	s	s & f	
11.	1	15	Gelding	Fell Pony	s & f	s & f	
12.	1	12	Gelding	TB X	s	s & f	
13.	1	15	Gelding	TB X	s & f	s & f	
14.	1	12	Mare	Welsh X	s	s	
15.	1	10	Gelding	TB X	s	s	
16.	1	14	Gelding	TB X	s	s	
17.	1	10	Mare	TB X			✓
18.	1	8	Mare	Welsh cross			✓
19.	1	7	Gelding	Cob cross			✓
22.	1	12	Mare	TB X	f	f	
21.	1	6	Gelding	Thoroughbred			✓
22.	1	10	Gelding	Clydesdale	f	f	
23.	1	10	Gelding	Cob X	f	f	
24.	1	6	Gelding	Welsh	f	f	
25.	1	11	Gelding	Cob X	f	f	
26.	1	7	Gelding	Welsh X	f	f	
27.	2	12	Gelding	Irish			✓
28.	2	18	Gelding	Thoroughbred			✓
29.	2	12	Gelding	Irish			✓
30.	3	12	Gelding	TB X			✓
31.	3	11	Gelding	TB X			✓
32.	4	13	Gelding	Cob X			✓
33.	4	7	Gelding	Irish			✓
34.	4	9	Gelding	Thoroughbred			✓
35.	1	12	Gelding	Welsh X		f	
36.	1	10	Gelding	Cob X		f	
37.	1	9	Mare	Welsh X		f	

Note: Saliva and faeces were collected from subjects according to their availability during the study. ‘S’ denotes that saliva was collected, and ‘f’ denotes that a faecal sample was taken.

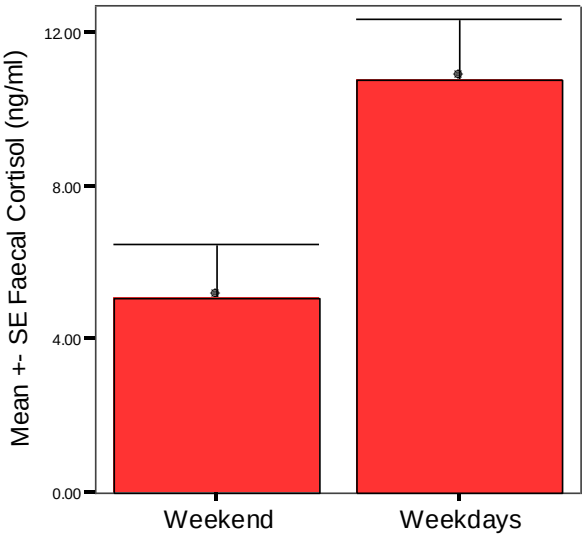

Figure 1.

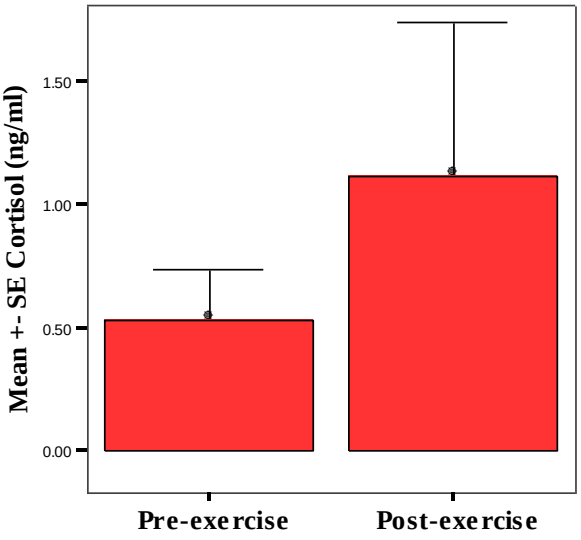

Figure 2.

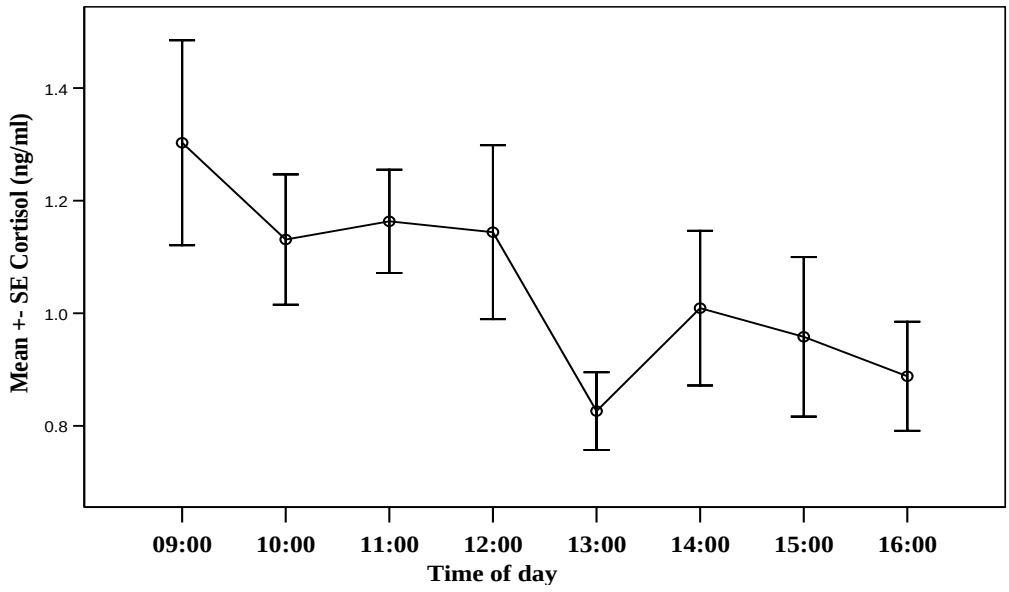
Mean +- SE Cortisol (ng/ml)
1.4
1.2
1.0
0.8
09:00
10:00
11:00
12:00
13:00
14:00
15:00
16:00
Time of day

Figure 3.

Table and figure captions

Table 1. Details of the subjects used for the assay validation and for the routine husbandry procedures.

Figure 1. The effect of weekday stabling and exercise on faecal cortisol concentrations of domestic riding horses (ng/ml) (N=18).

Figure 2. Salivary cortisol concentration of domestic riding horses 30 minutes before and after light exercise (N=8).

Figure 3. Diurnal variation of salivary cortisol in domestic riding horses between 0900h and 1600h (N=15).

Chapter three

A novel scale of behavioural indicators of stress for use with domestic horses.

Abstract

Behaviour scores (BS) offer non-invasive, objective and easy to use ways of assessing welfare in animals. Their development has; however, largely focused on behavioural reactions to stressful events, and little use of physiological measures has been made. The aim of the present study was to develop a scale of behavioural indicators of stress, for the purpose of welfare assessment in stabled domestic horses. To achieve this, behavioural and physiological data were collected form 32 horses that underwent routine husbandry procedures. Principal component analysis (PCA) of the behavioural and physiological data revealed three meaningful components that were used as the basis to the scale. Analysis of video clips of the horse's responses to the husbandry procedures was completed by a panel of equestrian industry professionals using a free choice profiling (FCP) methodology. The panel's results were added to the scale as was information from relevant literature. Salivary cortisol levels were significantly correlated with the BS confirming the scale was meaningful and reflected physiological stress. The scale offers an easy to use 'tool' for welfare assessment in horses, and reduces the need for physiological measures.

Introduction

Use of behaviour scores (BS) offer objective immediate methods of welfare assessment in animals (Minka et al., 2009). They have been used to measure stress levels for the purpose of welfare assessment in various species (horses: Visser et al., 2010; Munsters et al., 2011; cats: McCune, 1994; Kessler and Turner, 1997; McCobb et al., 2005; Dybdall et al., 2007; goats: Minka et al., 2009; cattle: Maria et al., 2004; Ostriches: Minka and Ayo, 2008). These scores have, however, been developed by focusing on expression of behaviour rather than making use of both behavioural and physiological indicators of stress.

Measurement of the stress response is best carried out using both behavioural and physiological measures (Broom, 1991; Mason and Mendl, 1993; Dawkins, 2003). This approach provides the best overall measurement of stress levels, and avoids drawing misleading conclusions that could be reached by taking just a single measure (Broom, 1991; Mason and Mendl, 1993; Dawkins, 2003). Use of BS has, therefore, been made as part of an integrated approach to measuring stress alongside physiological measures. Success at correlating BS with physiological measures has, however, been mixed. For example Minka et al. (2009) established that BS and physiological indices of stress in goats were related during the handling and loading of goats for transportation. In contrast McCobb et al. (2005) was not able to correlate BS with urinary cortisol in cats housed in traditional or enriched shelter environments.

The only scale of BS available for use with domestic horses was developed to assess whether or not horse and rider combinations were appropriate (Munsters et al., 2011). The scale was adapted from a scoring system used by Visser et al. (2010) to assess the temperaments of sports horses that were exposed to novel objects. The scale of BS compiled by Munsters et al. (2011) ranged between zero that described a completely relaxed horse, to 10 that depicted a very anxious horse. Although, when testing the scale, the BS were correlated with measures of heart rate variability (HRV), physiological measures were not used it’s during compilation.

The aim of the current study was, therefore, to develop a scale of BS that could be used to assess stress levels in domestic stabled horses. Our aim was to produce a scale that was simple quick and easy to use, where horse owners and behavioural scientists alike could provide horses with a BS according to behaviour exhibited for the purpose of measuring stress. Use was made of both behavioural and physiological measures during the scales' development, so the resulting BS also served as indices of physiology making them more robust and reliable.

The scale was developed using behavioural and physiological measures obtained from horses during naturally occurring daily routine husbandry procedures. Use of husbandry procedures, that would have taken place irrespective of the study, was considered an ethical approach to data collection rather than exposing horses to potentially stressful situations. A range of different types of mares and geldings of various ages were used for the study (n=32) with behavioural responses to the husbandry procedures being analysed in all animals. Physiological measures were collected from different animals to ensure a diversity of stress levels was sampled, from which to build the scale of BS.

In light of the recommendation to assess welfare using multiple measures (Broom, 1991; Mason and Mendl, 1993; Dawkins, 2003), analysis of the behavioural responses to the routine husbandry procedures was carried out using two different approaches. Behaviour was quantified using The Observer (Noldus Information Technology Software Ltd) for 12 of the horses, and both quantitative and qualitative assessment of the behaviour of all study horses was completed using the experimental free choice profiling (FCP) approach (Wemelsfelder et al., 2000; 2001). This approach enabled panel members to generate their own qualitative descriptions of behaviour observed and then quantify them according to their own subjective scales, rather than providing them with descriptive terms or pre-set rating scales to choose from.

We used both HR and salivary cortisol as physiological indices of stress to help develop the BS scale. Saliva was collected from 19 of the horses, and heart rate (HR) was recorded from 18 of the horses. Both indices of stress were used as their measurement could be achieved by non-invasive means, thus avoiding any further stress to the study horses. Both cortisol (Ralston et al., 1988; Toutain et al., 1995; McBride and Cuddeford, 2001; Covalesky et al., 1992; Stegaman and Jones, 1998) and HR (Reitmann et al., 2004; von Borell et al., 2007; Visser et al., 2008) are established indicators of stress in horses, and provide different measures of the stress response.

Method

Subjects used for the study

A group of 32 horses were used in this study. Nineteen of the horses were used for saliva collection for the purpose of cortisol measurement, with a further 10 of the horses used as a control group for this part of the study. Eighteen of the horses were used for measurement of HR, with a further 10 horses again used as a separate control group for this aspect of the study. Analysis of behaviour was completed from all 32 horses.

The horses used in the study consisted of various breeds of stabled mares and geldings kept in similar management and exercise regimes, at four different locations (a College yard, two different livery yards, and a Mounted Police unit). All of the horses were housed in individual stables on either straw or shavings bedding. They all received hay or haylage and water with up to two hard feeds i.e. mix or pellets, at around 0700h and 1600h. All horses were in light to medium work (receiving around two hours of exercise daily) throughout the study. When they were not being exercised all horses received limited (maximum duration of half a day) turnout to pasture daily, and remained in their usual daily management routine apart from undergoing routine husbandry procedures.

Husbandry procedures used for data collection

Horses were subjected to one of four different 10 minute husbandry procedures i.e. sound of electric coat clippers, social isolation, grooming and

exposure to the sound of fireworks played on C.D. Horses used for the grooming procedures were loosely held by a familiar handler, but all other horses were loose in their stables and had access to hay and water. A 10 minute period was deemed adequate to induce and measure a potential stress response, but not so long that habituation to the stressor should occur (Visser et al., 2001). Saliva was swabbed from 19 of the horses for cortisol analysis, and HR was measured in 18 of the horses. Only six of the horses had both physiological measures taken from them to ensure a good range of horses were used in the study from which to build the BS scale. Analysis of behavioural data were collected from all 32 horses and used to compile the BS scale.

Procedure 1 - sound of electric coat clippers

Horses were exposed to the sound of electric coat clippers (Heiniger Handy Clipper, Switzerland) turned on to maximum clipping velocity. Typical sound emissions from such clippers were 80.1 decibels. Clippers were switched on and held by hand in an adjacent stable to the study horse, with no interaction between the horse and researcher taking place.

Procedure 2 - social isolation

Horses were caught from the field and returned to their usual stable. This process took no longer than five minutes, and horses showed no resistance to capture. Horses were stabled in the absence of any other horses on the yard for 10 minutes. At the end of the social isolation period the horse's usual neighbouring horse was brought in from the field, and stabled next door to the study horse.

Procedure 3 - grooming procedures

A head-collar and lead rope was fitted to the horse, and a familiar handler held the lead rope approximately half way along its length to restrain the horse loosely. Mane combing and mane pulling (a procedure used to thin and shorten the mane by taking small sections of hair back combing them and pulling out the remaining long hairs) then took place.

Procedure 4 - the sound of fireworks played on a CD

Police horses were used for this procedure as it involved the sound of fireworks played on a compact disc (CD), which was used as part of riot training with Police horses. The CD player was situated on a table outside the horse's stable.

The husbandry procedures were carried out over a number of weeks in an ad hoc fashion, as the opportunities to collect data arose. Where the same procedures were carried out with a number of horses on the same yard e.g. exposure to the sound of electric coat clippers, one week was left between tests to minimise the effects of habituation on the horses that had not yet been sampled from.

Measurement of salivary cortisol concentrations during the husbandry procedures

Saliva was collected from the experimental group of horses (n=19) that underwent husbandry procedures to investigate the effects that such procedures had on stress levels. Saliva was collected 60 minutes and 30 minutes prior to the start of the husbandry procedures, and then at the end of the 10 minute procedure and at 10 minute intervals up to 40 minutes. Forty minutes was chosen to provide enough time for peak cortisol to be reached following the onset of the potential stressor (the husbandry procedure). Plasma cortisol peaks in horses 30 minutes post exercise stress (Foreman and Ferlazzo, 1996; Marc et al., 2000; Hamlin et al., 2000), and 20 minutes following restraint stress (Hydbring et al., 1996).

Saliva was collected from the control group of horses (n=10) to ensure that the swabbing procedure did not affect their stress levels. Collection took place at 60 minutes, 30 minutes, and 0 minutes before the husbandry procedure would have begun, and then at the same time intervals that the experimental group of horses had their saliva collected. An extra swab was taken from control horses to provide robust control measure.

Saliva was collected using sterilised flexi-swabs (Medical Wire & Equipment Co (Bath) Ltd) that were introduced into the corner of the horses' mouths first on the horse's left and then on the horse's right. The horses were allowed to manipulate the swabs using their tongues for approximately 20 seconds per introduction of the swab. The swabs were then placed into sterile 20ml plastic screw top containers, labelled and stored on ice until frozen at -20°C the same day to await cortisol extraction.

Saliva was extracted from the thawed cotton wool swabs by centrifugation using a Sorvall T.C. centrifuge (Thermo Scientific, Basingstoke, Hampshire, UK) for two minutes at 800g. The supernatant was then centrifuged using a Hettick Mikro 20 centrifuge (Tuttilgen, Germany) at 15,000g for two minutes. The supernatant was taken off using a pipette and frozen to await analysis. Salivary cortisol concentrations were quantified using a modified version of an EIA described by Smith and French (1997).

Measurement of heart rate (HR) during the husbandry procedures

HR was recorded from the experimental group of horses (n=18) horses that underwent husbandry procedures, to investigate the effects that such procedures had on levels of stress. HR was also measured from the control group of horses (n=10) in the absence of any husbandry procedures, to ensure that the method of recording HR did not cause stress to the horses. HR was recorded from both groups of horses at 60-second intervals for two minutes prior to the start of the husbandry procedure to provide a mean baseline HR. Recording of HR then continued at 60-second intervals for the first five minutes of the husbandry procedure for the experimental group of horses, and in the absence of a husbandry procedure for the control group.

HR was recorded using a Polar HR monitor (S610i) (Polar Electro, Öy, Kempele, Finland). The HR monitor consisted of an electrode belt that picked up the electrical activity of the horse's heart, with a transmitter attached enabling wireless transmission of the HR to a wrist watch receiver. The belt was fitted around the horse's thorax with both electrodes sited to the left-hand

side of the horse's thorax with one about 10cm below the withers and the other about 10cm behind the elbow over the heart. Warm water and electrode gel (The Wyke of Shifnal, Shropshire) was used to optimise contact between the horse's skin and the electrodes. The wrist watch receiver was taped to a leather strap fastened around the horse's neck. All horses were given 10 minutes to habituate to the equipment (Reitmann et al., 2004).

Behavioural analysis completed during the husbandry procedures

The behaviour of the subjects was recorded during all husbandry procedures using a Sanyo CCD/BW video camera (Sanyo Electric Co., Ltd, Osaka, Japan) secured at ceiling height in an appropriate position opposite the stable to gain an adequate field of view. The video camera was linked to a Mitsubishi HS-1024E time-lapse recorder (Osaka, Japan), set to three hour real time for recording of images onto three hour video tapes (BASF Vision Chrome Videocassette, BASF plc, Middlesex, U.K.).

Analysis of behaviour carried out by a panel of equestrian professionals

A panel of 13 professionals who had a working background with horses and held British Horse Society qualifications (minimum B.H.S. stage one - comprising of basic horse knowledge and practical care) was convened, and members were briefed on the nature of the study. They were asked to view a video of the initial two minutes of each horse's behavioural reaction to the husbandry procedures, and to provide a BS between zero and ten according to how stressed they perceived the horse to be. They were told that ten equated to an extremely stressed horse. The panel also had to describe using their own descriptive terms the horse's behaviour exhibited during the video. They finally had to state at which point on their subjective scale that they believed the onset of stress occurred in the horses undergoing the husbandry procedures.

Analysis of behaviour using The Observer

The first five minutes of the behavioural reactions exhibited during the husbandry procedures, for 12 of the horses, was also analysed using The Observer 5.0. Behaviours were recorded using a pre-defined ethogram based

on equine stable behaviour (Table 1). The ethogram had been compiled from six weeks of ad-hoc observation of race-horses and stabled riding horses, together with literature research (see Houpt, 1993; Winskill et al., 1996; McBride and Cuddeford, 2001; Strand et al., 2002; Heleski et al., 2002; Seaman et al., 2002; McDonnell, 2003)

Statistical analysis

All data sets were explored for normality and homogeneity of variance by means of Shapiro-Wilks' and Levene's tests respectively. Where data differed significantly from normal distribution non-parametric statistics were used. All statistical tests were two-tailed unless stated otherwise.

Changes in salivary cortisol measures obtained from the control horses (n=10) were investigated using a one factor repeated measures ANOVA. Changes in salivary cortisol during each of the husbandry procedures were explored by first calculating the median baseline cortisol concentration pre-husbandry procedure for each horse. The peak cortisol concentration following the end of the 10 minute procedure was then identified, and Wilcoxon Signed Rank tests were used to compare baseline and peak cortisol concentrations for each type of husbandry procedure. Data were then combined from all husbandry tests and baseline and peak cortisol titres compared using Wilcoxon Signed Rank test

Changes in HR measured from the control horses (n=10) were investigated using a Friedman test. Separate Friedman tests were used to explore changes in HR recorded during each of the husbandry procedures. HR data was then pooled from the husbandry procedures, and changes were again explored using Friedman.

Principal component analysis (PCA) was used to investigate whether there were any relationships between behavioural and physiological changes that took place during the husbandry procedures. 'Data reduction' was necessary to look for smaller sets of factors or components in the data (Pallant, 2004; Ennos, 2007) from which the scale of BS could be built. The percentage

change in cortisol concentration from the median baseline value to the peak concentration was calculated for each horse. This percentage, together with the percentage duration of all behaviours included in the ethogram underwent PCA.

PCA of the cortisol and behavioural data exhibited during the husbandry procedures revealed correlation coefficients of 0.3 and above (following Pallant, 2004). An oblimin rotation of three factor solution was used to reduce the number of variables into meaningful components (Pallant, 2004). Each behaviour and change in cortisol concentration received a score for each component denoting whether the behaviour was performed or not, or whether change in cortisol was relevant. A median BS was calculated for each horse used in the study (n = 32), as scored by members of the professional panel. The terms used by the panel to describe each horse's behaviour was pooled for horses with the same BS. Panel descriptions of behaviour were added to the relevant sets of factors or components revealed by the PCA, and the scale of BS for use with stabled domestic horses subsequently devised.

To investigate whether the devised BS scale reflected physiological stress, median BS as calculated from the professional panel, and peak salivary cortisol following the husbandry procedures were investigated using Spearman's Rank Order Correlation.

Results

Changes in physiological data collected from control horses were explored to ensure that the methods of data collection did not affect the horses. There were no changes in levels of salivary cortisol across the control study suggesting that the saliva swabbing was not stressful (Two-way ANOVA: $F_{7} = 0.82$, P = n.s.). There were, also, no significant changes in HR in the control study suggesting that the presence of the Polar heart rate monitor did not cause the horses any stress (Friedman test: $\chi^{2} = 7.36$, d.f. = 7, P = n.s.).

Changes in physiological data collected during the different types of husbandry procedures were explored to ensure that levels of stress induced did not differ significantly from each other. Salivary cortisol measures were explored for each husbandry procedure, and no significant differences in concentrations were found between measures taken before the husbandry procedure began and the peak concentration measured after the end of the procedure (Table 2). Measures of HR calculated before and during the different husbandry procedures, also did not reveal significant differences (Table 2).

When the data for the different husbandry procedures were analysed together, changes between baseline and peak salivary cortisol concentration showed a significant difference (Figure 1. Wilcoxon Signed Rank tests: Z = 13.29, P = 0.04, n = 18). A significant difference in HR measured before and during the husbandry tests when analysed together was also noted (Friedman test: χ^2 = 13.29, d.f. = 6, P = 0.04). These results suggest that routine husbandry procedures did elevate stress levels.

PCA of the percentage change in salivary cortisol from baseline to peak, and percentage duration of state behaviour exhibited during the husbandry procedures identified three components in the pattern matrix. They were labelled no stress (factor 1), low stress (factor 3) and medium stress (factor 2) according to the type of behaviour and change in cortisol identified (Table 3).

Median behaviour scores were calculated for the study horses (n=32), and ranged between one and eight. The terms used by the panel to describe each horse's behaviour was pooled for horses with the same BS, and panel descriptions of behaviour were added to the three components revealed by the PCA. The scale of behavioural indicators of stress for use with stabled domestic horses was subsequently devised (Table 4).

Descriptions used for horses with a BS of one and two were added to the component labelled no stress, as the mean score representing the onset of

stress as judged by the panel was three. Descriptions used for horses with a BS of three to seven were added to low and medium stress. The BS of five was used as the onset of medium stress, based on the type of behaviour included in the component extracted by the PCA. Descriptions used for horses with a BS of eight to ten formed a new category labelled high stress, because the PCA analysis did not include horses with a BS of this level. Relevant literature was also used to form this category as very few horses in the study were scored at this level.

A significant correlation existed between median BS and peak salivary cortisol concentration measured during the husbandry procedures ($r_s = 0.54$, $P = 0.02$, $n = 18$). This confirmed that the BS measured for individual horses was appropriate, and reflected physiological stress.

Discussion

The importance of using multiple measures to assess stress in horses

This study has resulted in the development of a scale of behavioural indicators of stress that can be used to measure stress levels in stabled domestic horses for the purposes of welfare assessment. An integrated approach to measuring levels of stress in the study horses was adopted by taking both physiological and behavioural measures. This ensured that the final scale was as accurate a representation of stress as possible having been developed using multiple measures, rather than by taking just a single measure of stress that could potentially be misleading (Broom, 1991; Mason and Mendl, 1993; Dawkins, 2003). It also enabled this study to build on previous methods used to develop scales of BS, which have made use only of behavioural analysis of the stress response (for example the cat stress score: Kessler and Turner, 1997).

By using both behavioural and physiological measures to develop the scale of behavioural indicators of stress, the BS also provide indices of underlying physiology. Changes in salivary cortisol concentration measured in response to the routine husbandry procedures were included as part of the PCA. Any association between changes in cortisol and behaviour exhibited in response

to husbandry was, therefore, included in the components identified by the PCA.

Levels of stress used to compile the scale of BS

The three components identified formed the basis to the final scale of BS. A common theme within the behaviour associated in each component was looked for to help identify what the component revealed about the horses' reactions to the husbandry procedures. The behaviour in component one suggested horses showed no reaction to the husbandry and, therefore, a stress response was not evident. This was supported by the association with change in cortisol revealed. Horses that were not deemed to be exhibiting a stress response remained standing at the front of their stables, with their head either down or looking around, and with their ears either pricked or scanning.

Behaviour associated in the other two components suggested that horses were exhibiting a stress reaction. Behaviours included in component three suggested a mild stress reaction, and so was used as the foundation to the low stress category on the scale of BS. Horses showing a low stress response did not reveal an association for where they stood in the stable, they spent time looking around, exhibited some ear flattening behaviour and tail swishing. Behaviour in component two included a stronger association between behaviour indicative of stress, such as showing a clear interest in what was happening in their surroundings with head and neck height above withers, and neck elevated above 45 degrees. Horses in this component also showed an increased level of activity in comparison to horses in the other categories of lower levels of stress, with walking and exploring behaviour being associated for the first time. Defecation was also associated in this component. These behaviours suggested that the horses exhibited a more acute stress response to the husbandry procedures, and so this component was used as the foundation to the medium stress category on the scale of BS.

Had further detailed behavioural categories been included in the ethogram used for The Observer analysis, or a method for reflecting the intensity of behavioural actions been used, further refinement in behavioural analysis

could have been achieved. For example tail swishing was associated with no and low stress horses. If the intensity of this behaviour had been identified, tail swishing to remove flies could have been distinguished from tail swishing associated with aggression for example. This would have helped improve the distinction between the behaviour included in each level of stress response.

Abnormal or stereotypic behaviour was included in each component extracted by the PCA. No stress horses showed an association with repetitive oral behaviour such as crib-biting, low stress horses exhibited weaving, and both low and medium stress horses carried out repetitive head movements such as head shaking or nodding. It has been suggested that performance of stereotypic behaviour may serve as a way of reducing stress levels, or as a way of horses' providing themselves with some sort of control over their environment (Cooper and Albentosa, 2005). This may explain the fact that horses perceived as experiencing no or low stress were exhibiting stereotypies. Weaving, which is indicative of chronic frustration in horses usually associated with attempts to gain social contact with other horses (Visser et al., 2008), was evident in medium stressed horses. This together with repetitive head movements suggests an increased level of frustration experienced by the horses in the low and medium stress groups.

The descriptions of horse behaviour exhibited in response to the husbandry procedures, as described by the professional panel were then added to the three categories of behaviour (no stress, low and medium stress). The horse's median BS was used to allocate behavioural descriptions to the appropriate stress level. The professional panel had identified a BS of three as the onset of stress, so horses with scores of one and two were added to the no stress level. A BS of five was used as the onset of medium stress based on an increased level of active behaviour being exhibited, and more interest in the horse's surroundings being evident. Horses with a BS of five and above were, therefore, added to the medium stress level. The behavioural descriptions of the few horses in the study that scored eight and above, were then transferred to form a new category on the scale labelled high stress. Relevant literature was also used to develop this category as

very few horses in the study were scored at this level. The behaviour indicative of a high level of stress response included horses described as agitated, fidgety, anxious, active, aggressive, or uncomfortable (McDonnell et al., 1999; Strand et al., 2002).

Defining the BS for measuring stress in horses

Each category of stress level was then sub-divided so that horses could be assigned an actual BS in the future when the scale was used in practice. Behavioural descriptions contained in each category incorporated actual body movements, as coded by The Observer, and subjective terms used by the professional panel. Both objective and subjective descriptions of behaviour were separated out to form the different BS in each stress level. For example in the low stress level horses with a BS of three exhibited behaviour seen in horses with a BS of one and two, but also showed occasional stereotypies, flattened their ears at times and defecated. They were also described as listening, interested and alert. Horses with a BS of four exhibited behaviour evident in the horses with lower BS but also showed pacing behaviour, approached potential stressors, swished their tails and were described as curious, unsettled and barging. Care was taken to ensure that a clear distinction in terms of behaviour exhibited, and descriptive terms used for horses with different BS could be made. Previous BS (see Munsters et al, 2011) have made use of subtle changes in behavioural reactions for different scores, so require a degree of personal judgement to decide on how much of a certain behaviour is being exhibited.

To ensure the final scale of BS was logical and measured increasing levels of behavioural and physiological stress, the relationship between behavioural and physiological changes in response to the husbandry procedures was investigated. Measures of salivary cortisol concentration following the routine husbandry procedures, and the median BS calculated for the same animal were seen to correlate. This indicated that the BS provided by the professional panel were accurate, and reflected increasing levels of physiological stress. This was important as previous work has found that physiological and behavioural measures do not always correlate (such as

urinary cortisol and behaviour exhibited by cats: McCobb et al., 2005), and this has been blamed on the individual variation in coping styles employed by animals under stressful conditions. A lack of correlation between behaviour and physiology may also be evident in mature animals that have learnt to suppress typical behavioural responses (such as horses when ridden suppressing the urge to flee from stressful situations: Munsters et al., 2011). The scale produced in this study did not appear to be affected by these factors.

Conclusion

The scale of behavioural indicators of stress developed in this study provides a quick and easy method of assessing the stress levels of domestic stabled horses. It was developed using both behavioural and physiological measures, so the final behavioural scores that make up the scale also provide indices of physiological change in response to stress. The relationship between behavioural and physiological changes inferred in the scale was further confirmed by the correlation seen between salivary cortisol and the same horses' BS. This, therefore, reduces the need to measure various physiological parameters separately to support the scale during its use, so makes it an appropriate measure that could be used by horse owners and behavioural scientists alike.

Acknowledgements

We are grateful to the horse owners for allowing us to collect behavioural and physiological data from their animals. We also appreciated the support given to us by the stable staff at Welsh college of Horticulture, Granary Livery, Belle Vue Livery, and Merseyside Mounted Police unit, and by undergraduate students from the University of Chester who helped with data collection during the study. We also thank members of the professional panel who gave up their time to contribute to this study. Thanks are also extended to Dr. Rob Coleman for his help with the cortisol assay, and to Dr. Joanna Hockenhull for her assistance with this study.

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Table 1.

Behaviour	Behavioural Description
Whole body	
Rolling	Drop to knees, roll onto side then back and perhaps all the way over then return to feet (adapted from Strand et al, 2002).
Lying	Lying with sternum in contact with ground surface, legs folded under the body or stretched out on side with legs stretched out (adapted from Heleski et al, 2002).
Standing at front	Standing with weight resting on 3 or 4 legs with one or both ears in front half of stable (adapted from Seaman et al, 2002).
Standing at back	Standing with weight resting on 3 or 4 legs with both ears in back half of stable (adapted from Seaman et al, 2002).
Walking	Four beat gait of forward movement (Heleski et al, 2002).
Trotting	Two beat diagonal gait of forward movement (Heleski et al, 2002).
Buck	A springing motion consisting of raising the hindquarters off the ground sometimes accompanied by a longitudinal twisting of the body (adapted from Mal et al, 1991).
Rear	Front legs raised off the ground with forehand higher than hindquarters (adapted from Heleski et al, 2002).
Barging	Forceful contact directed at a part of the stable either from a standstill or from any gait.
Scratching	Rubbing any part of the body against part of the stable or using hind foot or teeth to scratch part of own body (adapted from Heleski et al, 2002).
Pawing	Striking a vertical or horizontal surface or the air with forelimb (Seaman et al, 2002).
Kicking	One or both hind legs thrust backwards or to the side, contact with part of the stable may be achieved (adapted from Seaman et al, 2002).
Weaving	Lateral movement of the head, neck and shoulders from side to side in a rhythmic repetitive manner with alternation of the weight onto the contralateral foreleg with respect to the position of the head (adapted from McBride and Cuddeford, 2001).
Shake	Rapid rhythmic rotation of head, neck and upper body along the axis whilst standing with feet planted (McDonnell, 2003).
Stretch	Rigid extension of the limbs and arching of the neck and back (McDonnell, 2003).
Tail	
Raised	Fleshy part of tail outstretched horizontally or elevated above horizontal.
Neutral	Fleshy part of tail relaxed against body or moving slowly from side to side never raised to horizontal level.
Swish	Tail is flicked to one side and/or the other of the quarters.
Clamped	Fleshy part of the tail is forced close to the dock. Possible tensing of the quarters.
Defecation/ Urination	Elimination of faeces and urine
Neck	
Above withers	Eye level elevated above height of withers.
Below withers	Eye level parallel or below height of withers.
Over 45 degrees	Neck raised over 45 degrees
Ears	
Forward	Ears pricked up pointing forwards and stationary for three seconds or more.
Scanning	Ears moving back and forth at varying speeds.
Back	Ears apart or gently back and stationary for three seconds or more.
Flat	Ears pressed caudally against the head and neck (McDonnell and Haviland, 1995).
Mouth	
Eating/Drinking	Prehending, masticating or swallowing food or water (adapted from Winskill et al, 1996).
Exploratory	Lick, sniff or touch with muzzle or tongue parts of the stable or floor (adapted from Strand et al, 2002).

Self-Care	Non-ingestive behaviours involving the muzzle and teeth including allogrooming and swatting flies on body
Repetitive oral	A repeated, relatively invariant sequence of movements with no obvious function using the teeth or lips in contact with parts of the stable or own body (adapted from Mason, 1991). Includes cribbing, teethscraping, wood chewing, windsucking, biting, lipsmacking and self mutilation.
Yawn	Deep long inhalation with mouth widely open and jaws either directly opposed or moved from side to side (McDonnell, 2003).
Head	
Surveying	Head scanning through forty-five degrees or more.
Repetitive head movement	A repeated, relatively invariant sequence of movements with no obvious function (adapted from Mason, 1991) including movements of the head such as headshaking, nodding, bobbing and circling.

Notes to support categories of Mouth and Head Movements:

Repetitive Oral

- Cribbing – Horse grips onto a fixed object using incisor teeth, leans back onto hindquarters and contracts the strap muscle of the neck to bring the head into an arched position. Air is sometimes taken into the oesophagus to produce a grunting sound (McBride and Cuddeford, 2001).
- Teeth scraping – Lips are curled back to expose incisors. Lateral and corner incisors are scraped back and forth against side of solid object.
- Biting – Bite movement directed at or in contact with part of the stable.
- Lipsmacking – Incisors are kept shut whilst lips are opened and closed.
- Self-mutilation – Bite movement directed at own body usually the flanks or the chest and limbs (adapted from Houpt, 1993).
- Wood Chewing – Teeth are used to chew parts of the stable. Ingestion of wood may occur.
- Windsucking – Head and neck outstretched, mouth open slightly, air is taken into oesophagus.

Repetitive Head Movement

- Head Shaking – Head is shaken from side to side.
- Nodding – Head is moved up and down.
- Bobbing – Head performs a 'pecking action', where nose is only moved a small distance up and down.
- Circling – Whole head is circled to the left or right. Whole or half circles maybe exhibited.

Self Care

- Autogrooming – Nibbling, biting, licking or rubbing a part of the body (McDonnell, 2003)
- Swat Flies – Head is swung to hit insects on body or limbs (adapted from Strand et al, 2002).

Table 2.

	Husbandry Procedures			
	Sound of fireworks	**Social isolation**	**Sound of electric clippers**	**Grooming procedures**
HR	$\chi^2 = 5.82$, d.f. = 6, P = n.s	Data not collected	$\chi^2 = 5.89$, d.f. = 6, P = n.s	$\chi^2 = 7.89$, d.f. = 6, P = n.s
Salivary cortisol	Z = -1.60, P = n.s., n = 3	Z = -1.52, P = n.s., n = 7	Z = -0.73, P = n.s., n = 6	Z = -0.54, P = n.s., n = 3

Table 3.

	Component		
	1 – No stress	2 – medium stress	3 – low stress
Change in cortisol concentration	-0.648		
Standing at the front	0.886		
Standing at the back	-0.806	-0.374	
Walking		0.802	
Scratching		0.863	
Weaving			0.740
Eating and drinking	-0.797		0.304
Exploring		0.860	
Self-care	0.446		-0.402
Repetitive oral behaviour	0.364		-0.661
Other mouth behaviour	0.817		
Surveying with head	0.602	-0.310	0.342
Repetitive head behaviour		0.838	0.477
Other head behaviour	-0.600		-0.395
Tail raised		0.861	
Neutral tail posture	-0.817		
Other tail behaviour	0.816		
Neck height above withers		-0.931	
Neck height below withers	0.307	0.449	-0.346
Neck height over 45 degrees		0.936	
Ears pricked	0.592	0.550	
Ears scanning	0.885		
Ears back	-0.879	-0.309	
Ears flat			0.740
Other ear behaviour			-0.345
Tail swishing	0.635		0.349
Defecation		0.811	
Eigenvalues	8.23	7.04	3.01
% variance accounted for	30.51	26.07	11.13

Table 4.

Stress level	Behaviour score	Behavioural indicators
No stress	1	Standing at the front of the stable, looking around or head below wither height, eating. Ears pricked, back or slowly scanning, tail still or gently swishing. Some repetitive oral behaviour. *Horse described as:* Horse calm, unconcerned, relaxed, quiet, listening, accepting.
	2	*Behaviour exhibited for previous BS plus:* Walking. *Horse also described as:* Horse alert and watching.
Low stress	3	*Behaviour exhibited for previous stress level plus:* Occasional weaving behaviour, box walking and repetitive head movements. Ears occasionally flattened. Defecation. *Horse described as:* Listening, interested, alert.
	4	*Behaviour exhibited for previous BS plus:* Pacing. Approaching potential stressors e.g. noise from outside the stable. Repeated tail swishing. *Horse also described as:* Curious, unsettled, barging.
Medium stress	5	*Behaviour exhibited for previous stress level plus:* Scratching against stable walls or fittings, pawing at ground with front legs. Nostrils flared. Repeatedly looking around. Tail raised. *Horse described as:* Restless, showing tension in the body, fidgeting when still.
	6	*Behaviour exhibited for previous BS plus:* Approaching and retreating away from potential stressors. Stopping eating to focus on potential stressor. *Horse also described as:* Jumpy, easily startled.
	7	*Behaviour exhibited for previous BS plus:* Keeping away from potential stressors and remaining still to focus on them. *Horse described as for previous BS.*
High stress	8	*Behaviour exhibited for previous stress level plus:* Repeated performance of stereotypic behaviour e.g. weaving, box walking repetitive head movements. Stamping of hind feet. Snorting. *Horse described as:* Very unsettled and alert.
	9-10	*Behaviour as exhibited for previous BS.* *Horse also described as:* Agitated, fidgety, anxious, active, aggressive, uncomfortable (McDonnell et al., 1999; Strand et al., 2002).

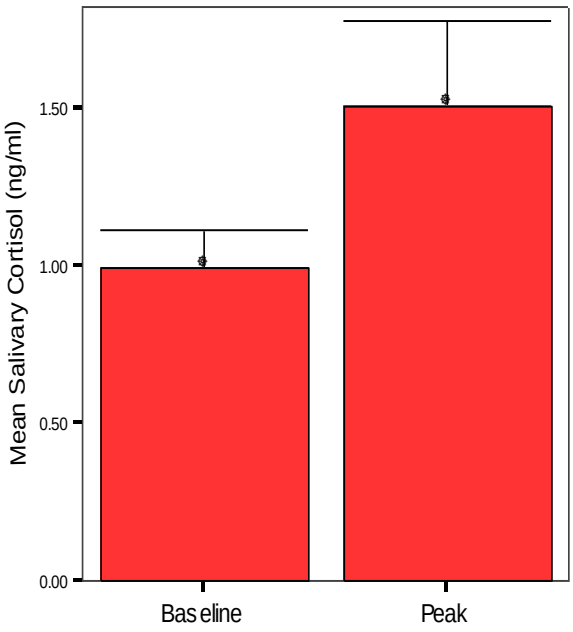

Figure 1.

Table and figure captions.

Table 1. The ethogram used for The Observer configuration.

Table 2. The effect of husbandry procedures on the HR and salivary cortisol concentrations of stabled domestic horses.

Table 3. Pattern matrix for PCA of the cortisol and duration of state behaviour recorded during the four different routine husbandry procedures using oblimin rotation of a three factor solution.

Table 4. A scale of behavioural indicators of stress in domestic stabled horses, as revealed by principal component analysis (PCA) and behavioural assessment completed by a professional panel.

Figure 1. Mean salivary cortisol (ng/ml) ± 1.0 SE before the husbandry procedures (Baseline) and at peak following the procedure (n=19).

Chapter four

Assessing the effectiveness of a novel method for measuring stress levels in individually stabled and group housed horses

Abstract

Behaviour scores (BS) offer easy to use, immediate, non-invasive, and economic measures of stress in animals. The scale of behavioural indicators of stress for use with domestic horses used in this study (Young et al., MS) was compiled using behavioural and physiological measures of stress, and, therefore, offered a novel approach to measuring the stress levels of horses. The goal of the current study was to use these BS to assess stress in horses that were stabled individually and group housed. The effectiveness of the BS at measuring levels of stress was assessed, and compared with the effectiveness of measuring stress in the same horses using established physiological measures of stress, i.e. heart rate variability (HRV) and faecal cortisol. BS and physiological measures were taken from ten horses stabled individually, and nine that were group housed (GH). Data were also taken from four horses that were moved from individual stabling to group housing for a period of three weeks. Use was made of an independent scorer for scoring the behaviour in an attempt to increase the objectivity of findings. Lower levels of stress were recorded in GH horses as revealed by the BS, but physiological indices showed no significant difference between horses in either type of housing. BS and physiological measures did not reveal a change in stress levels following the move to GH, but BS showed a significant decline over the three weeks of GH. Physiological indices remained unchanged during the period of GH. The stress levels of horses in both housing environments were, finally, investigated whilst horses were waiting to be fed. BS results suggested that the GH horses experienced lower levels of

stress whilst waiting to be fed, but physiological results largely suggested no difference between the two environments. Results revealed by the BS throughout the study were supported by relevant literature. BS measured in horses over the three weeks of GH, also correlated with HRV (represented by average R-R interval, which was the time recorded between successive heart beats) measured in the same animals. It was concluded, therefore, that the scale of behavioural indicators of stress was an effective method of measuring stress levels in horses. It appeared to be more sensitive than the physiological measures used, as they did not reveal significant results possibly due to the small sample sizes. The scale of BS, therefore, provides a promising novel approach to measuring the stress levels of stabled horses that requires further investigation to build on findings made in this preliminary study.

Introduction

Behaviour scores (BS) have been used as a way of assessing stress for the purpose of welfare assessment in various animals (horses: Visser et al., 2010; Munsters et al., 2011; Young et al., MS; cats: McCune, 1994; Kessler and Turner, 1997; McCobb et al., 2005; Dybdall et al., 2007; goats: Minka et al., 2009; cattle: Maria et al., 2004; Ostriches: Minka and Ayo, 2008). They offer easy to use, on the spot methods of welfare assessment (Minka et al., 2009; Young et al., MS), and could provide a novel way of assessing the stress levels of horses kept in different types of housing. Traditionally, scales of BS were developed by focusing on behavioural elements (for example the cat-stress-score: McCune, 1994; Kessler and Turner, 1997). The scale of behavioural indicators of stress compiled by Young et al. (MS) used in this study, however, made use of both behavioural and physiological measures during its development. This meant that the final BS provided indices of physiological status

This study aimed to investigate whether the scale of BS was effective at measuring stress levels in individually stabled (IS) and group housed (GH) horses. It also aimed to investigate the effectiveness of measuring stress in the same horses using established physiological measures of stress, i.e. heart

rate variability (HRV) and faecal cortisol. Faecal cortisol provides an index of cortisol concentration within the horse's body over a 24 hour time period (Mostl and Palme, 2002), and HRV assesses the balance of activity between the sympathetic nervous system (SNS) and the parasympathetic nervous system (or vagal system), so is deemed an effective way of assessing on-going stress in the horse (Bachmann et al., 2003; Reitmann et al., 2004b; von Borell et al., 2007). Results obtained using behaviour scores were assessed in the context of the physiological results, and relevant published literature.

We know that housing horses in a group environment has been linked to a reduction in their stress levels, and it has been suggested that as a result this improves their welfare (Keeling et al., 2007). Studies have indicated GH to be beneficial to horse welfare by identifying better social skill development in young horses that were GH as opposed to socially isolated (Houpt et al., 1984; McCall et al., 1985; 1987; Søndergaard and Ladewig, 2004; Harewood and McGowan, 2005; Visser et al., 2008), by reducing stress in weanling horses (Heleski et al., 2002), and by noting how horse-human relationships developed more easily when horses were GH (Søndergaard and Ladewig, 2004).

It was, therefore, predicted the stress levels of the IS horses in this study would be higher than in GH horses, and this would be reflected by higher BS and faecal cortisol concentrations, and lower measures of HRV (Luescher et al., 1991; Cooper et al., 2000; McAfee et al., 2002; Visser et al., 2008). In other studies when behaviour was compared between GH and IS stabled horses, those housed as a group exhibited a lower amount of abnormal behaviour suggesting reduced stress levels (Cooper and McGreevy, 2002; Bachmann et al., 2003; van Dierendonck et al., 2004; Fremstad et al., 2008). Comparisons made between physiological measures taken from GH and IS horses showed those stabled defecated more, had higher levels of heart rate (HR) together with lower levels of heart rate variability (HRV), and increased cortisol concentrations (Bagshaw et al., 1994; Harewood and McGowan, 2005, Visser et al., 2008).

As part of this study, a small number of horses were moved from IS to GH to enable a preliminary investigation into the effects of moving horses from IS to GH. It was predicted that the move to the new type of housing would be stressful (Irvine and Alexander, 1994), and this would be evidenced by elevated BS and cortisol, with lower measures of HRV. This preliminary study was important because fears relating to potential injury when group housing horses for the first time prevents many horse owners from keeping their horses in this way, despite the benefits offered to their welfare in the long-term. Horses are often aggressive on meeting other new horses (Knubben et al., 2008; Hartmann et al., 2009), but this is usually just display behaviour thereby reducing potential injuries (Jørgensen et al., 2009).

The final part of the study aimed to investigate the effects of waiting to be fed on the stress levels of the IS and GH horses. Waiting to be fed has been seen to increase stress in horses (Alexander and Irvine, 1998; McGreevy, 2004) so was used as a known stressor in this study. This was carried out because the social companionship provided by GH has been linked to lower stress levels in these animals (Houpt et al., 1984; McCall et al., 1985; 1987; Heleski et al., 2002; Harewood and McGowan, 2005; Visser et al., 2008). It was predicted that the stress levels of the horses accustomed to GH would be lower in the presence of the stressor than the horses that were IS.

Method

Subjects and experimental design

Fifteen horses kept at four different locations were used in this study (Table 1). Data was collected on a Tuesday and Wednesday during one week, from 10 of the horses that were IS and had been for a minimum of two weeks. A minimum of two weeks has been identified as necessary to enable horses to habituate fully to a new environment (Irvine and Alexander, 1994; Harewood and McGowan, 2005). Four of these horses were then moved to a barn (9.5m by 9.5m) and GH in pairs (Table 1). Data was collected on a Tuesday and Wednesday each week from these four horses over a three week period. During the third week of the study, data was collected on a Thursday and Friday from a further five horses that had been GH for longer than a two week

period. Two of these horses were housed as a pair, and the other three horses were kept as a small group in a barn of approximately 9.5m by 9.5m. Data was therefore collected from 10 IS horses, four horses that had been moved from IS to GH, and from a total of nine horses accustomed to GH (Table 1).

All horses were turned out to pasture either over night or during the day (Table 1), and turn out groups remained the same throughout the study with GH horses being turned out together. The horses were in light/medium work receiving one to two hours of exercise daily, apart from horses five, 14 and 15 who were rested for reasons other than ill health. All horses received hay or haylage and water when IS or GH, and were given a hard feed in a bucket e.g. mix or pellets, on being brought in from the field and prior to being turned out.

Data collection

Data were collected for two hours daily between 0900h and 1000h, and 1700h and 1800h. The first data collection hour was carried out once horses had been brought in from the field and had finished their hard feed (post-feed), and the second hour was before horses received their second feed of the day (pre-feed) and were turned out to grass. BS, measures of HRV and faecal cortisol concentrations were collected to compare the stress levels of IS and GH horses, but only BS and measures of HRV were used to measure the effects of waiting to be fed on horses in both housing environments.

Measuring the behaviour of stabled and group housed horses

Behaviour was scored live during both hours of data collection at five minute intervals using the scale of behavioural indicators of stress for domestic horses (Young et al., MS). This enabled an hourly median BS for each horse to be calculated for both hours. Scoring was carried out by a trained independent scorer who worked in the equestrian industry to increase the objectivity of the BS.

Physiological data

Recording heart rate variability

Heart rate was sampled from all horses during both data collection hours for measurement of heart rate variability (HRV). HR was recorded using a Polar heart rate monitor (RS800) (Polar Electro, Öy, Kempele, Finland) programmed to record R-R data which enabled beat-to-beat (R-R) intervals to be recorded. An electrode belt with a transmitter was fitted around the horse's thorax, and within the belt were two electrodes that picked up the electrical activity of the heart. The electrodes were fitted to the left-hand side of the horse's chest with one about 10cm below the withers and the other about 10cm behind the elbow over the heart. Warm water and electrode gel (The Wyke of Shifnal, Shropshire) were used to optimise contact between the horse's skin and the electrodes. Electrical activity was transmitted wirelessly to a wrist watch receiver attached to a leather strap around the horses' neck. All equipment was fitted to the horse over thirty minutes prior to data collection to enable the horse to become accustomed to wearing the equipment (following Eager et al., 2004). The electrical activity was stored in files within the wrist watch computer and was then downloaded via a Polar Interface (Polar Electro, Öy, Kempele, Finland) to a computer where analysis was enabled using the Polar Protrainer 5™ Equine Edition (Polar Electro, Öy, Kempele, Finland).

Filtering heart rate variability data

The two hours of data collection were sampled for HRV analysis from the overall data recorded, and filtered for erroneous values. Errors in the data appeared in the heart rate curves as sudden peaks in the R-R data. R intervals refer to the time between successive heart beats (von Borell et al., 2007), and errors could have occurred either due to measurement errors, or as a result of extra heart beats. A moderate filter power with a minimum protection zone of 6 beats per minute (bpm) was used, and the number of errors in the data checked following the automatic error correction. The correction method was repeated until errors were reduced to zero before analysis was carried out

Faeces collection for cortisol measurement

A faecal sample was collected during the first hour of data collection (between 0900h and 1000h) from all horses in both housing environments, on day two of the two consecutive data collection days, and on the following day. These collection days accounted for the 24 hour time lag of cortisol excretion in faeces (Palme et al., 1996). Where possible faecal collection took place at similar times each day to minimise confounds due to circadian variation (see Millspaugh and Washburn, 2004)

Faecal samples were collected following defecation by removing a pinch of faeces from the centre and each sides of the pile, in an attempt to gain a representative sample of any hormone present. Gloves were worn during sample collection. This method aimed to overcome the problem of uneven hormone distribution in the faecal bolus recorded for some species (see Millspaugh and Washburn, 2004). Samples were then placed into sterile 20ml plastic screw top containers, labelled, and stored on ice until frozen at -20°C the same day to await cortisol extraction.

Faecal cortisol extraction and measurement

Following defrosting 10g of each faecal sample was weighed, placed in a foil container, and dried at 40˚C in a drying oven for two days. Each dried sample was then ground in a pestle and mortar to increase the surface area of the faecal matter and sifted through a fine wire mesh to remove fibrous material. A 0.2g sample of the resulting powder was mixed vigorously with 3ml of 90% methanol (M/4053/17, Fisher, Loughbourgh, UK) through agitation using a vortex for one minute, and then by being shaken for three hours in a shaking incubator at 25˚C (Stuart Scientific, UK). The samples were centrifuged using a Sorvall T.C. centrifuge (Thermo Scientific, Basingstoke, Hampshire, UK) at 800g for 15 minutes. The resulting supernatant was removed and poured into another glass test tube, and the methanol was evaporated off using compressed oxygen free nitrogen gas (N_2) administered using a Pierce Reacti-Therm Heating Module (Pierce, Rockford, Illinois, USA) for approximately 40 minutes at 40^{o}C.

The samples were reconstituted in 1ml of EIA phosphate buffer saline solution (PBS) (PBS – 5.42g $NaH_2PO_4H_2O$, 8.66g Na_2HPO_4 [anhydrous], 8.7g NaCl, 1.0g BSA [RIA Grade Albumin Bovine] and 1L dH_2O, pH 7.0, stored at 4°C). To ensure mixing; test tubes were agitated using a vortex for 3 minutes, and then the solutions were frozen at –20˚C to await assay.

Following extraction, cortisol was assayed in triplicate by Cortisol Enzyme Labelled Immunosorbant Assay (ELISA) using the competitive antigen capture method (modified from Smith and French, 1997). Following the assay the plates were washed three times to remove unbound components, tapped dry and 100µl of Tetramethyl benzidine (TMB) substrate solution (Biovet, Quebec, Canada) added to all wells. After a further hour of incubation at 25˚C in the dark, the reaction was stopped using 50µl of 1M phosphoric acid added to all wells. The concentration of free-cortisol in each well was determined from absorbance read at 450nm using Dynex Revelation Software (v4.22) calibrated against commercially prepared cortisol standards, and corrected for any absorbance read from the control wells. A final concentration of cortisol (ng/ml) was provided for each horse by calculating the mean concentration from the replicates.

Statistical analysis

All data sets were explored for normality and homogeneity of variance by means of Shapiro-Wilks' and Levene's tests respectively. Where data differed significantly from normal distribution non-parametric statistics were used. All statistical tests were two-tailed unless stated otherwise.

BS of stabled and GH horses

A mean weekly BS for pre- and post-feeding over the two days of data collection was calculated using data from all horses. A weekly mean BS was then worked out by calculating the mean of the weekly pre-and post-feeding score.

The mean weekly BS of the horses accustomed to IS and GH (week three data) was compared using an independent samples t-test as the data were

normally distributed. This investigated whether the stress levels of either group of horses who were accustomed to their housing differed.

The mean weekly BS of the IS and GH horses in week one of GH was then compared using Mann-Whitney U test for independent groups as only four of the horses were moved from IS to GH. This investigated whether the move to GH had affected stress levels of the four horses concerned. The effect of moving to a new housing environment was further investigated by examining the change in BS over the three weeks of GH using the Kruskal-Wallis test for independent groups. A test for independent measures was necessary as BS measured from the five further GH horses used in the study were added to week three data. It was presumed that the four horses moved to GH would be accustomed to their environment by then. Post hoc testing was completed using Mann-Whitney U tests.

The effects of waiting to be fed on the BS of the IS and GH horses in week three was analysed. Post and pre-feeding mean BS were compared for horses in each housing condition using a paired samples t-test. This confirmed whether waiting to be fed was stressful to the horses concerned. Post-feeding BS was then compared between the two housing conditions using an independent samples t-test, and the same repeated for the pre-feeding BS.

HRV of stabled and GH horses

The number of heart beats over each hour of data collection was provided by Polar Protrainer 5™ Equine Edition, and the mean weekly measures across all horses calculated following the method used for the BS. The average bpm were then calculated by dividing the number of heart beats recorded over the hour by 60 minutes. The average R-R interval (milliseconds) was also provided by Polar Protrainer 5™ Equine Edition, and the mean weekly measures across all horses calculated. Statistical analysis of the number of heart beats and average R-R interval (ms) was completed using the same tests as applied for the BS.

Analysis of faecal cortisol data

Mean faecal cortisol concentration (ng/ml) was calculated for each horse over the two days that faeces were sampled. Faecal cortisol concentrations of IS horses were compared to horses that were GH in week one of data collection, using the non-parametric Mann-Whitney U test for independent groups, and in week three of GH using an independent samples t-test. The effect of moving to the new housing environment was further investigated by examining the change in cortisol concentration over the three weeks of GH using the Kruskal-Wallis test for independent groups.

Correlations between behavioural and physiological data

The mean BS was calculated across all horses sampled during each week of GH, and this was correlated against the mean weekly value for each physiological measure taken. Spearman Rank correlation was used to test for linear association between the behavioural and physiological data.

Results

BS of IS horses and horses GH housed over three weeks

Mean BS were calculated for horses housed individually and GH over the three week period (Table 2). The stress levels of the IS horses were compared to horses accustomed to GH (as represented by week three data) using mean BS (Table 2). A significant reduction in BS was evident in the GH horses (Independent samples t-test: $t_{17} = 2.70$, P=0.02) suggesting their stress levels were lower than those measured in the IS animals.

The impact on stress levels of moving a number of IS horses to GH was investigated by comparing the mean BS of the IS horses to the GH animals in week one. No significant difference in BS was revealed (Mann Whitney U test for independent groups: $Z_{10, 4} = -0.57$, n.s.) suggesting there was no difference in levels of stress as measured by BS. The mean BS did, however, show a trend towards a significant decrease over the three weeks of GH suggesting a reduction in stress levels (Figure 1. Kruskal-Wallis test: K = 5.83, 2 d.f., P=0.05). Post hoc tests revealed a significant decrease in mean

BS between weeks two and three (Mann-Whitney U test: for independent groups: $Z_{4,9}$ = --2.16, P=0.03, n=13.)

The effect of waiting to be fed was investigated using the mean BS of horses in both housing environments (Table 3). When post and pre-feeding BS were compared in IS horses, a significantly higher mean BS was measured in horses waiting to be fed than post-feeding (Paired samples t-test: t_7= 3.57, P=0.01). This indicated that waiting to be fed was stressful to IS horses. There was no significant difference between post and pre-feeding BS in the horses accustomed to GH (Paired samples t-test: t_6= 1.33, n.s.).

Mean BS of IS and GH horses were compared post-feeding, and revealed significantly higher scores in the stabled horses suggesting elevated levels of stress (Figure 2. Independent samples t-test: t_9= 3.34, P=0.01). Pre-feeding BS of IS and GH horses also revealed significantly higher levels, again suggesting IS horses to be more stressed than those that were accustomed to GH (Figure 3. Independent samples t-test: t_{13}= 2.39, P=0.03).

HRV measured in individually stabled horses and horses GH housed over three weeks

Mean measures of HRV were calculated for horses housed individually, and GH over a three week period (Table 4). The mean heart rate (HR), as revealed by numbers of heart beats, showed no statistically significant difference between horses IS or accustomed to GH (Independent samples t-test: t_{12} = 1.16, n.s.). The same was true of the average R-R interval compared between horses in both housing environments (Independent samples t-test: t_{12} = 1.87, n.s.). These results suggest that horses accustomed to GH had comparable levels of stress to those stabled individually.

No significant difference in the mean HR or average R-R interval was found when comparing horses stabled individually, with those that had been moved to GH (Mann Whitney U test for independent groups: HR: $Z_{10, 3}$ = -1.18, n.s., R-R interval: $Z_{10, 3}$ = -1.35, n.s.). This suggested that the move to GH had not

impacted on stress levels, although HR was higher in the GH horses and the average R-R interval had decreased. HRV measures also showed no significant difference over the three week period of GH despite a decline in HR being evident, together with an increase in average R-R interval (Kruskal-Wallis test: HR: K = 0.01, 2 d.f., n.s.; R-R interval: K = 1.28, 2 d.f., n.s.).

Comparisons between pre and post-feeding measures of HR in both IS horses and those accustomed to GH (Table 5) showed no significant difference (HR: Paired samples t-test: Individually stabled: t_7=0.14, n.s.; GH: t_5=1.20, n.s.). The same was also true of the average R-R interval measured in both housing environments (R-R interval: Paired samples t-test: Individually stabled: t_7=0.77, n.s.; GH: t_5=2.21, n.s.).

Comparisons between post and pre-feeding measures of HR between the two types of housing also revealed no significant differences (Independent samples t-test: Post-feeding: t_{14}=0.72, n.s.; pre-feeding: t_{12}=0.60, n.s.). The average R-R interval showed no significant difference between horses in both housing types post-feeding (Independent samples t-test: t_{14}=0.55, n.s.), but did show that horses that were accustomed to GH had significantly lower average R-R intervals pre-feeding suggesting they were more stressed than the stabled animals (Independent samples t-test: t_{12}=2.56, P=0.03).

Faecal cortisol concentrations measured in horses stabled individually and GH over a three week period

There was no statistically significant difference in the mean faecal cortisol concentration of horses stabled individually or accustomed to GH (Table 6. Independent samples t-test: t_{12} = 1.16, n.s.), suggesting that the stress levels of both groups of horses were comparable.

No significant increase in mean faecal cortisol was evident in the horses that had been moved from IS to GH (Table 6. Mann-Whitney U test for independent groups: $Z_{10, 4}$= -0.71, ns). Faecal cortisol concentrations also remained constant over the three weeks of GH, suggesting that stress levels

did not differ significantly across this period (Kruskal-Wallis test: K = 0.45, 2 d.f., n.s.).

Behavioural and physiological data

A significant linear association between mean BS and average R-R interval was identified over the three weeks of GH (Spearman Rank correlation: r_s = -1.00, P = 0.00, n = 3) (correlation was significant at the 0.01 level). As BS decreased average R-R interval increased, suggesting a decrease in the stress levels of the GH horses. BS was not correlated with any other physiological measures over the three weeks (Spearman Rank correlation: BS and faecal cortisol: r_s = -1.00, P = 0.67, n = 3; BS and HR: r_s = -1.00, P = 0.67, n = 3).

Discussion

The study used two very different approaches, the scale of BS and established physiological measures i.e. HRV and faecal cortisol, to quantify the levels of stress in horses stabled individually and GH. The assessment of stress of horses in both housing environments was achieved using a three pronged approach. Stress was measured in horses that were accustomed to both types of housing, stress was investigated in horses that had been moved from IS to GH, and the effects of a known stressor, waiting to be fed, on the stress levels of horses was explored in both housing environments. The two approaches did not reveal the same results, which highlights the importance of using a multivariate approach to measuring stress in animals for the purpose of assessing welfare. The BS provided biologically meaningful results that were supported by relevant literature and, therefore, suggested them to be a sensitive and accurate measure.

Comparing levels of stress in horses accustomed to IS and GH

The behaviour of horses accustomed to IS and GH was scored using the BS scale, and results suggested that GH horses experienced lower levels of stress than those that were IS. This supported published findings that have claimed GH to reduce the levels of stress in horses (Lebelt et al., 1998; McBride and Long, 2001; Cooper and McGreevy, 2002; Bachmann et al.,

2003; van Dierendonck et al., 2004; Fremstad et al., 2008). Measures of HRV and faecal cortisol concentrations did not show a significant difference between the two types of housing though, and so did not support the findings revealed by the BS. This may have been because some of the GH horses had only been kept in this way for a little over two weeks (following Irvine and Alexander, 1994; Harewood and McGowan, 2005), and may not have been fully accustomed to GH. Had further measures been taken over longer period of time, physiological indices of stress may have revealed the reduced levels of stress suggested by the BS. Results of the first part of the study, therefore, suggested that BS provided an easily observable immediate measure of stress, and provided results that supported relevant literature.

The impact of moving horses from IS to GH on levels of stress

BS and physiological measures did not suggest that moving horses from IS to GH had any impact on their stress levels. The findings of this second part of the study did not, therefore, support the prediction that stress levels would increase in horses moved to the new housing environment, as has been seen in other work (for example Irvine and Alexander, 1994; Schmidt et al., 2010). This may have been because the stress levels of the horses were already heightened as a result of coping with the confinement and social isolation of IS (Harewood and McGowan, 2005; Cooper and Albentosa, 2005; McCall, 2006), and because GH has been linked to a reduction in stress levels (e.g. Keeling et al., 2007). Further research into the effects of moving horses to GH using larger sample sizes than was able in this preliminary study is suggested, to help allay the fears of horse-owners about introducing their animals to GH.

Levels of stress were seen to decrease over the three weeks of GH following the move from IS, but a significant decrease was only revealed by the BS. HR did decrease during each week of data collection, with average R-R interval increasing accordingly, but such changes were not significant. The biggest drop in BS occurred between weeks two and three, suggesting that any group dynamics were beginning to be resolved by then reducing stress (Lehmann et al., 2006). It was also important to note that group structure

remained the same over the three weeks of the study, and this has been seen to reduce stress in GH horses by lessening the risk of aggression and injury (Zeitler-Fecht, 2004; van Dierendonck et al., 2004; Knubben et al., 2008). Once again the scale of behavioural indicators appeared to be sensitive enough to provide results that supported relevant literature. It was assumed that larger sample sizes were necessary to obtain significant physiological results due to large individual variation.

The effects of waiting to be fed, on the stress levels of IS and GH horses

The effects of waiting to be fed on the stress levels of IS and GH horses were, finally, investigated. Waiting to be fed has been seen to increase stress levels in horses (Alexander and Irvine, 1998; McGreevy, 2004), possibly through a build up of motivation in the animal as it anticipates forthcoming changes (Fraser and Broom, 1997). This was supported by the BS obtained from the IS horses pre-feeding, but not from those that were GH as no difference between pre- and post-feeding BS were evident. It could, therefore, be assumed that the social companionship provided by GH helps to reduce the physiological consequences of exposure to potential stressors (Houpt et al., 1984; McCall et al., 1985; 1987; Heleski et al., 2002; Harewood and McGowan, 2005; Visser et al., 2008). Measures of HR did not, however, reveal a difference between stress levels of horses before or after feeding in either housing environment. A significant difference between R-R intervals was, however, noted between horses pre-feeding. A lower average R-R interval was recorded in GH horses which contradicted the BS result. It suggested GH horses to be more stressed then those stabled individually prior to feeding. Horses subjected to physical or emotional stress experience greater SNS activity accelerating HR and reducing HRV (Reitmann et al., 2004b) and thus the R-R interval. This result, therefore, highlights the need for using an integration of multiple measures to assess stress levels of horses for the purpose of welfare assessment.

The results of the final phase of the study supported the notion that the scale of behavioural indicators of stress was a useful method of assessing the stress levels of stabled horses. It provided results that were in line with

predicted outcomes and relevant literature. In order to further investigate the validity of the BS scale, however, BS was correlated with physiological indices over the three weeks of GH. BS and average R-R interval showed a strong correlation over the three weeks. BS declined as the average R-R interval increased confirming a reduction in levels of stress, and suggesting that the BS scale was measuring stress levels in accordance with measures of the autonomic nervous system (ANS).

Conclusion

This study revealed that the scale of behavioural indicators of stress comprising of BS, was an effective method of measuring stress levels in IS and GH horses. Results gained through using this novel approach to measuring stress supported relevant literature, and produced biologically meaningful even when used with small sample sizes. The use of BS provided easily observable, immediate measures of stress that were non-invasive, economical, and could be used by a trained horse owner. Use of the scale of BS seemed to be more sensitive than the physiological measures, where significant results were not obtained possibly due to small sample sizes. The scale of behavioural indicators of stress, therefore, provides a promising method for measuring the stress levels of stabled horses that requires further investigation to build on findings made in this preliminary study.

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Table 1.

	Location	Gender	Age	Turn out time	Type of housing		Horses moved from IS to GH
					Individually stabled	Group housed	
1	1	gelding	12	Night	✓	✓	✓
2	1	gelding	11	Night	✓	✓	✓
3	2	mare	11	Night	✓		
4	2	mare	9	Night	✓		
5	2	mare	30	Night	✓		
6	2	gelding	21	Night	✓		
7	2	gelding	12	Day	✓		
8	2	gelding	19	Day	✓		
9	2	mare	7	Day	✓	✓	✓
10	2	mare	6	Day	✓	✓	✓
11	3	mare	11	Night		✓	
12	3	gelding	12	Night		✓	
13	3	mare	12	Night		✓	
14	4	mare	12	Night		✓	
15	4	mare	12	Night		✓	

Table 2.

Individually stabled horses (N=10)	Group housed horses		
	Week 1 (n=4)	Week 2 (n=4)	Week 3 (n=9)
1.69	1.75	1.63	1.31

Table 3.

Horses	Individually stabled horses (N=10)		Group housed horses (week 3) (N=9)	
	Post-feed	**Pre-feed**	**Post-feed**	**Pre-feed**
1.	1.23	No score	1.08	1.31
2.	1.31	No score	1.62	1.85
3.	1.04	1.73		
4.	1.62	1.23		
5.	1.23	1.85		
6.	1.31	1.96		
7.	1.65	2.69		
8.	1.58	2.35		
9.	1.96	2.00	1.23	1.96
10.	1.58	1.65	1.12	1.58
11.			1.23	1.00
12.			1.04	1.00
13.			1.27	1.20
14.			1.12	No score
15.			1.08	No score
Mean score	**1.45**	**1.93**	**1.20**	**1.41**

Table 4.

	Individually stabled horses (N=10)	**Group housed horses**		
		Week 1 (n=4)	**Week 2 (n=4)**	**Week 3 (n=9)**
Number of heart beats (bpm)	39.13	44.10	43.10	41.35
Average R-R interval	1,598.92	1,374.29	1,436	1,473.79

Table 5.

	Individually stabled horses (N=10)		Group housed horses (N=9)	
	Post feed	**Pre feed**	**Post feed**	**Pre feed**
Number of heart beats (bpm)	39.75	38.5	42.28	40.41
Average R-R interval (ms)	1,574.7	1,623.125	1,444	1,503.58

Table 6.

Horse	**Individually stabled horses (N=10)**	**Group housed horses**		
		Week 1 (n=4)	**Week 2 (n=2)**	**Week 3 (n=7)**
1.	7.55	7.56		
2.	11.67	9.84		
3.	6.27			
4.	4.55			
5.	5.48			
6.	4.38			
7.	11.48			
8.	5.17			
9.	7.33	6.53	9.73	7.90
10.	4.78	4.89	6.82	6.26
11.				9.17
12.				7.09
13.				8.12
14.				6.76
15.				7.23
Mean	**6.86**	**7.21**	**8.28**	**7.50**

Key: Data not collected

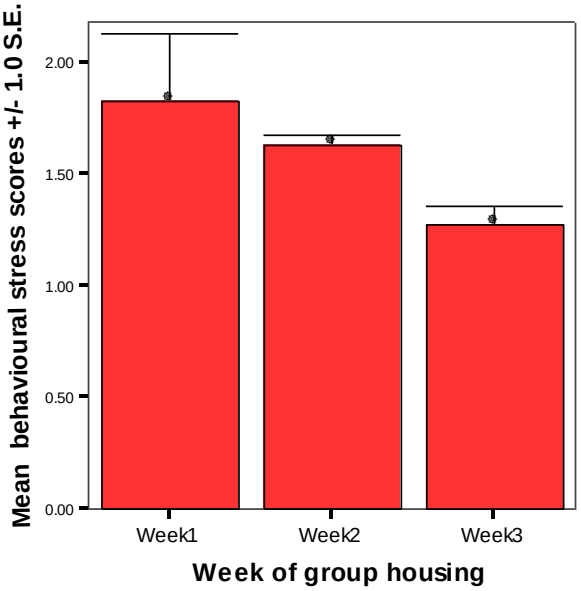

Figure 1.

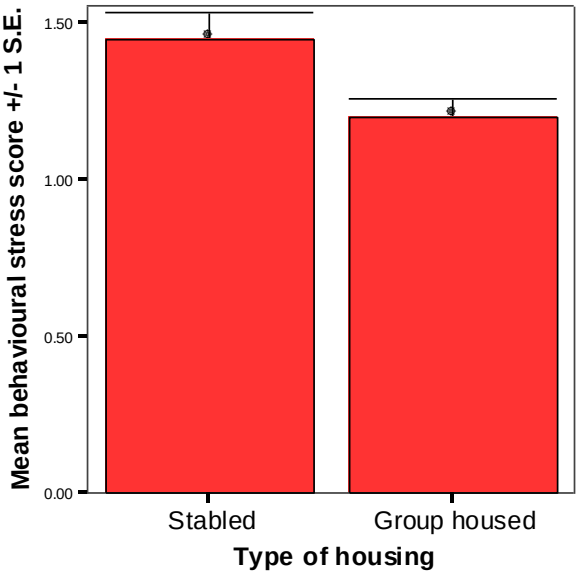

Figure 2.

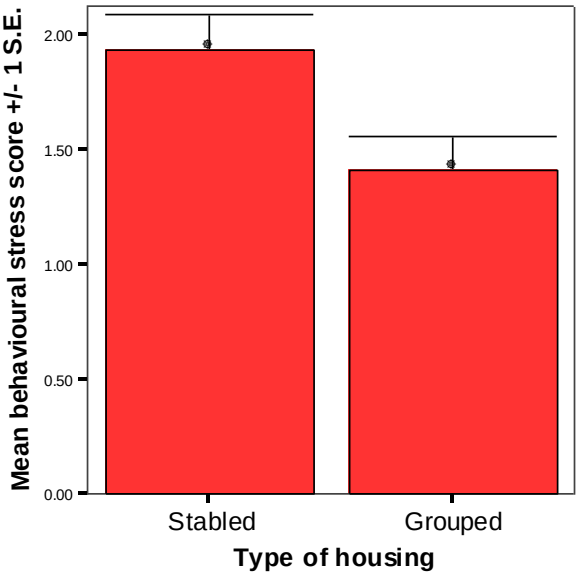
Mean behavioural stress score +/- 1 S.E.
2.00
1.50
1.00
0.50
0.00
Stabled
Grouped
Type of housing

Figure 3.

Table captions

Table 1. Details of the study horses (N=15).

Table 2. Mean BS measured from horses stabled individually and GH over a three week period.

Table 3. Mean BS post and pre-feeding measured from stabled and GH horses.

Table 4. Mean measures of HRV recorded from horses stabled individually and GH over a three week period.

Table 5. Mean measures of HRV post and pre-feeding recorded from horses stabled individually and group housed.

Table 6. Mean faecal cortisol concentrations measured in horses stabled individually and GH over a three week period.

Figure captions

Figure 1. Mean BS measured from horses over three weeks of GH.

Figure 2. Mean BS measured post-feeding in individually stabled and GH horses.

Figure 3. Mean BS measured pre-feeding in individually stabled and GH horses.

Chapter five
Final discussion and conclusions

The three main objectives of this study were first assessing whether or not modern husbandry procedures elevated stress levels in domestic horses, second developing a scale of behavioural indicators of stress that measured stress in horses for the purpose of welfare assessment, and third investigating whether or not the devised scale was effective at measuring horses' stress levels.

5.1 The effect of modern husbandry practices on the stress levels of domestic horses

The way we manage modern domestic horses is increasingly being scrutinised (McGreevy, 2007), and much criticism is levied at what were considered to be accepted practices such as individual stabling (Luescher et al., 1991; Cooper et al., 2000; McAfee et al., 2002; Visser et al., 2008). The first study in this research project (chapter two) aimed to contribute to the growing field of horse welfare research, by examining the effects of various husbandry practices on the stress levels of domestic horses. This was something not widely considered in the horse welfare literature and would, therefore, provide a novel contribution. Stress was measured using cortisol concentration in both horse saliva and faeces, and the validation of an assay for quantification of cortisol in both mediums in the horse also formed part of the study and thus further added to the field of horse welfare research.

It was evident from the study that exercising horses increased their stress levels, and it was assumed that individual stabling also contributed to the stress experienced. These findings supported relevant published literature that states that exercise increases stress (for example identified by Malinowski et al., 1993; Zobba et al., 2011), and that links stabling in particular to behavioural changes typical of elevated stress levels (Bachmann et al., 2003; Parker et al., 2008; Hockenhull and Creighton, 2010), and physiological changes associated with the stress response (Harewood and

McGowan, 2005; Visser et al., 2008). Lower levels of stress were reported in horses that were rested and turned out to grass, so it was suggested that working horses would benefit from periods of turn out to pasture and rest when possible. The value of turn out to pasture and free exercise has been noted in relevant literature (Rose-Meierhöfer et al., 2010; Werhahn et al., 2011), but on a practical level may not be possible for all horse owners due to restricted access to pasture land and constraints placed on turn out at commercial livery yards. This, therefore, increases the need for alternative methods of housing horses to be investigated so that opportunities for horses to move around, interact with other horses, and forage for food are provided.

The study also looked at the effects of short-term husbandry procedures such as exposing the horse to the noise of electric coat clippers, and to short bouts of social isolation. These practices were typical of the types of husbandry methods used with horses today, but their effects on stress levels are not widely documented within the horse welfare literature. The finding of research carried out for this thesis was that husbandry methods do elevate stress levels, but this was not seen in this initial study. This first study did, however, recommend that the effects of these types of routine management practices were further investigated, because College riding horses had been used in the study and they were typically exposed to a variety of management techniques daily so may have been more habituated to them than other groups of horses.

5.2 Measuring levels of stress in domestic horses

The assessment of stress levels in animals is best carried out by measuring both behavioural and physiological changes so that an integration of measures is provided to enable a robust conclusion to be reached (Broom, 1991; Mason and Mendl, 1993; Dawkins, 2003). By taking only a single measure of stress the results could be misleading, and incorrect conclusions drawn.

This idea underpinned the second study carried out in this research project (chapter three). A scale of behavioural indicators of stress for use with domestic horses was developed in the study for the purpose of welfare

assessment. The scale comprised of behaviour typically exhibited by horses that were experiencing elevating levels of stress. Subjective words that described the horses' behaviour at the various levels of stress were also included, as the scale was not just intended for use within behavioural science. It was expected that it may be of use to the typical horse owner wishing to monitor their own horse's well-being. The scale of behaviour scores (BS) built on similar scales that had previously been devised for other animals (horses: Visser et al., 2010; Munsters et al., 2011; cats: McCune, 1994; Kessler and Turner, 1997; McCobb et al., 2005; Dybdall et al., 2007; goats: Minka et al., 2009; cattle: Maria et al., 2004; Ostriches: Minka and Ayo, 2008), by making use of both behavioural and physiological changes typical of the stress response. The final BS provided indices of physiological change in response to stress and was, therefore, deemed a robust measure of stress in horses.

To develop the scale, stress levels were measured in horses that were undergoing routine husbandry procedures. The husbandry procedures were similar to those used in the first study, and included the sound of electric clippers, social isolation, grooming procedures, and for the Police horses used in the study the sound of fireworks played on a CD. This enabled the effects of short-term husbandry procedures on the stress levels of horses to be further examined. There was no significant difference in the amount of stress induced by the different husbandry practices, and when examined together it was revealed that the procedures did elevate horses' stress levels. It can, therefore, be concluded that the overall level of stress experienced by domestic horses arises not just from exercise or how the horses are housed, but is also contributed to by short-term management practices that the horse is exposed to on a daily basis. Such a finding requires further research so that procedures that put welfare at a particular risk can be identified, and modified or avoided where possible.

The scale of BS was intended to be a quick and easy, on the spot measure of stress in horses for the purpose of welfare assessment. Having been devised using physiological measures, the scale reduced the need to quantify other

physiological parameters separately to support the scale during its use. Difficulty was faced during this research at obtaining participants, particularly when the need to gather physiological samples was necessary. All samples were intended to be non-invasive, but even the process of taking a saliva swab was rejected by the owners of some competition horses where their animals could be subjected to doping tests, for example in horse-racing. It was hoped, therefore, that use of the scale of behavioural indicators of stress would increase the ability to measure stress in a diversity of horses.

5.3 Using the scale of behavioural indicators of stress to measure levels of stress in domestic horses

To test the effectiveness of the scale of behavioural indicators of stress at measuring stress levels in domestic horses, it was used to measure stress in individually stabled (IS) and group housed horses (GH) (chapter four). As mentioned previously IS of horses has been seen to elevate horses' stress levels and GH has been linked to a reduction in stress (Houpt et al., 1984; McCall et al., 1985; 1987; Heleski et al., 2002; Harewood and McGowan, 2005; Visser et al., 2008). There is increasing pressure today on land available that can be used to turn horses out to grass on, due to urbanisation and the cost of land for sale (Henderson, 2007). As a result many horses, particularly those in more urban areas, remain stabled for long periods of time with their only form of exercise being controlled, such as when ridden (Henderson, 2007; Werhahn et al., 2011). This is likely to impact on both their health and welfare, and so the need for alternative methods of housing such as in groups is increasingly important in today's society.

Results generated by the scale of BS were compared to measures of heart rate variability (HRV) and faecal cortisol obtained from the same animals. The scale of BS was easy to use having been implemented by an independent scorer, provided immediate measures of stress, and results supported relevant literature suggesting GH to reduce stress levels. It was also deemed to be a sensitive measure, as significant results were obtained even with the small sample sizes used in the study. The physiological results were not significant, possible due to the small samples and the individual variation

evident between animals. The scale was, therefore, concluded to provide an effective method for measuring the stress levels of horses housed in different types of housing.

5.4 Analysis of the methods used in the research

An integrated approach to measuring stress in horses was deemed necessary to provide a robust measure of stress that a single measure may not have revealed (Broom, 1991; Mason and Mendl, 1993; Dawkins, 2003). This approach was adopted in compiling the scale of behavioural indicators of stress for use with domestic horses. Levels of stress were measured by recording heart rate (HR), and by assessing cortisol concentration in response to husbandry procedures. Behaviour was analysed using The Observer, and through the use of free choice profiling (FCP) completed by a panel of equestrian professionals.

HR was not affected by use of the Polar heart rate monitor used for recording electrical activity of the horse's heart, and salivary cortisol was not affected by the swabbing procedure used to collect saliva from the horses' mouths. Both measures of physiology did not differ significantly between the different husbandry procedures used for measuring the potential stress response, but this may have been due to the small sample sizes involved with the different procedures. In order to investigate the effects of different methods of short-term husbandry procedures further studies need to recruit larger samples of horses. Both HR and salivary cortisol did, however, increase significantly when the effects of all of the husbandry procedures were investigated together, and so both were deemed suitable measures of short-term stress response. HRV has, however, been documented as a measure of stress response that is perhaps more suited to the assessment of on-going stress (Bachmann et al., 2003; Reitmann et al., 2004b; von Borell et al., 2007), so it was used in the final study of this research in preference to HR. Salivary cortisol was also concluded to be a better measure of short-term stress, since it demonstrates only a short time to reach peak concentration (approximately 30 minutes following exercise stress: Marc et al., 2000; Hamlin et al., 2002; and 65 minutes after semen collection: Lebelt et al., 1996), was a subject to

diurnal variation (Irvine and Alexander, 1994; Alexander et al., 1996; Lebelt et al., 1996), and was vulnerable to the effects of environmental stimulants (for example alterations in housing :Irvine and Alexander, 1994; social stress: Alexander and Irvine, 1998; or anticipation of forthcoming changes: Alexander and Irvine, 1998). Faecal cortisol was documented to be more suited to the assessment of on-going stress (Mostl and Palme, 2002), so it was chosen for use in the final study of this research.

The Observer provided a useful way of establishing the duration of state behaviours, and the rate of event behaviours exhibited by the horses in response to the husbandry procedures. It required detailed configuration prior to its use, which was achieved in this research by compiling a comprehensive ethogram of stable behaviour over six weeks of ad hoc study. The ethogram and thus the configuration still had the potential for inclusion of more behavioural elements enabling more detailed behavioural analysis to take place. It may also have benefited from the use of 'modifiers' that would have enabled the intensity of certain behaviours to be depicted. This would have helped distinguish a behaviour exhibited as a result of stress, from the same behaviour being carried out as routine self-care behaviour for example tail swishing to remove flies, or tail swishing as a result of aggression. Behavioural coding using The Observer is a detailed process though, and the use of more behavioural elements would give rise to further potential error. To over come this during this research each behavioural category, such as tail behaviour, was coded separately thus requiring each horse's video to be run six times for coding to be completed. This has obvious implications for using The Observer with larger sample sizes.

The FCP method (Wemelsfelder et al., 2000; 2001) was used by a panel of equestrian professionals to analyse the horses' behaviour in response to the husbandry procedures. The method enabled them to assign descriptions of each horse's behaviour, together with a number representing the level of stress they thought the horse exhibited. A range of descriptive terms were used to depict horse behaviour, and they together with the quantification of the stress level, corresponded well between panel members. Panel

membership was achieved on a voluntary basis for the research, so not all members were available to view every horse's behavioural response to the husbandry procedures. The method would have been improved had all members viewed all video clips, and if inter-observer reliability had been calculated between panel members to ensure that there was no one whose analysis differed significantly.

Associations between the physiological and behavioural analysis of the horses' reactions to the husbandry procedures were investigated using principal component analysis (PCA). This method of data reduction enabled smaller sets of factors or components in the data (Pallant, 2004; Ennos, 2007) to be established from which the scale of BS was built. The method proved to be useful in revealing three components, no stress, low stress and medium stress. These components were added to using the professional panel descriptions of behaviour which associated appropriately. The final phase in developing the scale involved the use of relevant literature to form the upper level of the scale, since the husbandry procedures induced little evidence of high levels of stress. To differentiate between different levels of stress seen within the four categories of stress making up the scale, BS were added. The method of assigning the BS made use of the scores measured by the professional panel, but some subjective judgement had to be used. Every effort was, however, made to distinguish between likely behaviour exhibited at each level of stress, and between the subjective terms included at each level.

5.5 Implications of the study findings

The findings of this study are applicable to all individuals responsible for looking after horses. The effects of typical methods of modern husbandry were investigated to see if they impacted on stress levels. Findings supported published claims that exercise and stabling elevated stress levels, but the study also revealed that short-term practices such as exposure to the noise of clippers and grooming have the potential to cause stress levels to increase. Such a finding has major importance to horse owners as it means that horses may be repeatedly stressed on a daily basis. This finding is not widely documented in the literature and warrants further research. If horses are

exposed to repeated bouts of short-term stress from daily husbandry procedures, and in some cases in addition to other on-going stressors, then their levels of welfare may rapidly diminish.

These findings raise the question as to whether horse owners and yard mangers should regularly monitor their horse's stress levels. The health of horses is checked on a daily basis, often just by briefly looking over the horse, but it is suspected that horse's stress levels are only considered when the horse shows immediate fear or excitement. The scale of behavioural indicators of stress offers a quick and easy way of monitoring stress. Owners could provide their horses with a BS, which would enable any fluctuations in stress to be noted. This could be particularly useful to those responsible for managing competition horses, such as race-horses, where even minor changes in stress levels have the potential to impact on performance. By monitoring levels of stress horse-owners could be informed about the effects of any changes in their horse's management, environment, exercise or training, before the changes jeopardise their horse's welfare.

5.6 Wider implications of the study findings

The research identified additional areas of study that would further contribute to the field of equine welfare research. More research into the effects of how we manage domestic horses on a daily basis is clearly necessary. A range of husbandry procedures need to be examined and their impact on stress levels investigated. Where necessary techniques may require modification, and for some horses techniques may be best avoided.

Alternative methods of housing horses' needs to be further researched. Individual stabling clearly does not complement how the horse evolved, and with the increasing urbanisation of horses (Henderson, 2007) access to turn out to grass may be further reduced. The implementation of methods such as open barn systems, which combine GH with access to sand paddocks (Rose-Meierhöfer et al., 2010) may offer a compromise by meeting the welfare needs of the horse, whilst coping with the restrictions placed on horse owners by limited access to land available. Further research into GH is, however,

necessary to allay owners' fears about introducing their horses to groups. The preliminary findings in this study did not encounter any evidence of aggressive interaction that would have warranted concern when mixing horses in a GH system, when horses had previously been turned out to grass together. Indeed, the move to GH from IS did not impact on the horses' stress levels at all. This was, however, a preliminary study and group sizes were small. Further work in this area is vital and ought to be a research priority.

The scale of behavioural indicators of stress offers a novel method for measuring the stress levels of stabled domestic horses, and could be applied to the areas of potential research identified here. Accurate methods for measuring horse welfare that avoid the need to obtain physiological samples that have the potential to be invasive, expensive to analyse, and require the expertise of highly trained individuals are surely welcomed. The scale of behavioural indicators of stress devised in this study represents a significant step forward in BS development, by providing indices of physiology and thus offers an integrated measure of welfare.

5.7 Final comments and conclusions

This study has contributed to research into the causes and impacts of stress on domestic horses. It has identified that even short-term, and what horse owners may perceive as accepted methods of looking after horses, can actually cause them stress. A much needed quick and easy to use method for measuring stress levels in horses was, therefore, developed as part of the study and was successfully used to measure the stress levels of IS and GH horses. The scale of behavioural indicators of stress was sensitive enough to yield significant results in the study, where established physiological method of measuring on-going stress could not. The scale of BS, therefore, offers great potential for measuring stress levels in a range of studies, where obtaining large sample sizes or physiological measures prove problematic. The scale was developed by integrating behavioural and physiological changes in response to husbandry techniques, so the resulting BS provides indices of physiological changes in response to stress. This reduces the need to measure supporting physiological parameters and, therefore, makes the

scale appropriate for use by horse owners. Findings from the research project raised the question as to whether horse's stress levels should be monitored in a similar way to daily health checks, to avoid health, welfare and performance levels being jeopardised. Use of the scale of BS would enable horse owners to be able to do this, and to make changes to their horse's environment and management before they impact on the horse. The study findings, therefore, made a unique and valuable contribution to horse welfare research, by adding to what is currently known about the causes of stress to domestic horses, by devising a technique for measuring stress levels in horses, and by evaluating the technique's effectiveness at measuring the stress levels of horses in different housing environments.

APPENDICES

Animal Ethics Committee letter of approval.

University of Chester

Dear Tamsin,

The Animal Ethics Committee has reviewed your application entitled '*Measuring stress levels in domestic horses by investigating the effects of housing type* 'at our meeting on the 4th of May 2010. Overall, the committee members were satisfied that the appropriate ethical considerations were undertaken with respect to your research plans.

The committee is grateful for your efforts in considering the ethical implications of your research and wishes you best of luck in your investigations.

Sincerely,

Professor Colleen M. Schaffner
Chair, Animal Ethics Committee

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SUPPORTING PAPERS

Hughes, T., Creighton, E. & Coleman, R. (2010) Salivary and faecal cortisol as measures of stress in horses. Journal of Veterinary Behaviour: Clinical Applications and Research. 5, 1: 59-60.

SALIVARY AND FAECAL CORTISOL AS MEASURES OF STRESS IN HORSES

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Valid measures of stress are needed in horses to determine over-all stress levels and to identify stress triggers, used to ensure management is kept within the animals' ability to cope and welfare is not compromised. Levels of circulating cortisol reflect HPA-axis activity, and excretion into saliva and faeces allow non-invasive sampling. We validated an enzyme-linked immunoassay (ELISA) for horse salivary and faecal cortisol and validated these as indicators of acute and over-all stress levels in riding horses. Saliva was swabbed every 30-minutes over three days in N=15 horses with eight in light exercise. Faeces were collected from N=9 working horses on stabled workdays and at rest at grass for three consecutive weeks. Immunological validity of the ELISA was demonstrated by high specificity, accuracy, precision and sensitivity. Biological validity of salivary cortisol was demonstrated by diurnal decline and elevation post-exercise both mirroring known patterns in plasma cortisol; and by a trend towards elevation following 10 minute exposure to a known stressor. Faecal cortisol was biologically validated by decline between working and rest days. Large individual differences in assay values were found and not all individuals followed the group means. Salivary cortisol was labile, and although it has a close temporal relationship to circulating cortisol, measures may be confounded by environmental disturbance, pulsatile release patterns and diurnal rhythm. Careful sampling protocols are therefore needed. Faecal cortisol as an index of circulating cortisol has a 24-hour time lag to excretion, and collection protocols must

evenly sample total faecal mass due to uneven hormone distribution and be frozen immediately post-excretion to avoid degradation. With careful sampling, salivary cortisol may be used to measure acute stress responses to identify stress triggers, and faecal cortisol may be used to compare over-all stress levels over longer-term conditions.

Key words: horse, cortisol, saliva, faeces, stress